





Digitized by the Internet Archive  
in 2010 with funding from  
University of Toronto

CUNNAUGHT  
RESEARCH



BINDING LIST DEC 15 1927.

CORNBAGHT LABORATORY  
RESEARCH DIVISION



# THE JOURNAL OF HYGIENE



CAMBRIDGE UNIVERSITY PRESS

C. F. CLAY, MANAGER

LONDON: FETTER LANE, E.C. 4



H. K. LEWIS & CO., LTD., 136, GOWER STREET, LONDON, W.C. 1

WILLIAM WESLEY & SON, 28, ESSEX STREET, LONDON, W.C. 2

CHICAGO: THE UNIVERSITY OF CHICAGO PRESS

BOMBAY, CALCUTTA, MADRAS: MACMILLAN & CO., LTD.

TORONTO: J. M. DENT & SONS, LTD.

TOKYO: THE MARUZEN-KABUSHIKI-KAISHA

*All rights reserved*

# THE JOURNAL OF HYGIENE

EDITED BY

GEORGE H. F. NUTTALL, M.D., PH.D., SC.D., F.R.S.

QUICK PROFESSOR OF BIOLOGY IN THE UNIVERSITY OF CAMBRIDGE

IN CONJUNCTION WITH

JOHN S. HALDANE, M.D., LL.D., F.R.S.

LATE READER IN PHYSIOLOGY IN THE UNIVERSITY OF OXFORD

CHARLES J. MARTIN, M.B., D.Sc., F.R.S.

DIRECTOR OF THE LISTER INSTITUTE, LONDON

J. C. G. LEDINGHAM, M.B., D.Sc.

BACTERIOLOGIST-IN-CHIEF, LISTER INSTITUTE, LONDON

G. S. GRAHAM-SMITH, M.D.

UNIVERSITY LECTURER IN HYGIENE, CAMBRIDGE

MAJOR GREENWOOD, M.R.C.P. (LOND.)

MEDICAL OFFICER (MEDICAL STATISTICS), MINISTRY OF HEALTH

VOLUME XVIII. 1919-20

168944.

26.1.2

CAMBRIDGE  
AT THE UNIVERSITY PRESS

1920

RA

421

J88

V. 18-19

cop. 2



# CONTENTS

## NO. 1 (APRIL)

	PAGE
GRAHAM-SMITH, G. S. Some Factors influencing the Actions of Dyes and Allied Compounds on Bacteria. (With Plate I and 6 Charts)	1
BROWNING, C. H. and GULBRANSEN, R. The Testing of Antiseptics in Relation to their Use in Wound Treatment . . . . .	33
WOOD, DENYS R. Recent Advances in the Differentiation of Lactose-fermenting (Gas-producing) Bacilli, with special reference to the Examination of Water and Food Products . . . . .	46
GRIFFITH, A. STANLEY. The Cultivation of <i>Spirochaeta icterohaemorrhagiae</i> and the Production of a Therapeutic Anti-spirochaetal Serum . . . . .	59
MACKIE, T. J. The Atypical Dysentery Bacilli . . . . .	69
ARKWRIGHT, J. A., BACOT, A., and DUNCAN, F. MARTIN. The Association of <i>Rickettsia</i> with Trench Fever. (With Plates II and III) .	76
EWART, R. J. The Influence of the Age of Parent at Birth of Offspring upon the Development of Eye Colour and Intelligence—A Correction . . . . .	95

## NO. 2 (AUGUST)

TULLOCH, W. J. Report of Bacteriological Investigation of Tetanus carried out on behalf of the War Office Committee for the Study of Tetanus. (With 7 Diagrams) . . . . .	103
FAIRLEY, N. HAMILTON. The Laboratory Diagnosis of Typhus Fever. Further Observations on the value and on the significance of the Weil-Felix Reaction . . . . .	203

## NO. 3 (OCTOBER)

CLELAND, J. BURTON and BRADLEY, BURTON. Further Experiments in the Etiology of Dengue Fever. (Clinical notes by MACDONALD, W.) (With 9 Charts) . . . . .	217
KENNAWAY, E. L. and WRIGHT, A. M. Two Instances of Human Sera showing Abnormal Anti-complementary Power . . . . .	255

	PAGE
McLEOD, J. W. Observations on the Cultivation of Typhoid and Paratyphoid Bacilli from the Stools with special reference to the Brilliant Green Enrichment Method . . . . .	260
EAGLETON, A. J. The Bacterial Content of the Air in Army Sleeping Huts, with special reference to the Meningococcus. (With 1 Diagram) . . . . .	264
CLELAND, J. BURTON and CAMPBELL, A. W. An Experimental Investigation of an Australian Epidemic of Acute Encephalo-myelitis .	272
DUDGEON, LEONARD S. On the Effects of Injections of Quinine into the Tissues of Man and Animals. (With Plate III) . . . . .	317
SHEARER, C. Studies on the Action of Electrolytes on Bacteria. Part I. The Action of Monovalent and Divalent Salts on the Conductivity of Bacterial Emulsions. (With 8 Charts) . . . . .	337

#### NO. 4 (FEBRUARY)

HORT, EDWARD C. The Cultivation of Aerobic Bacteria from Single Cells. (With 1 Text-figure) . . . . .	361
HORT, EDWARD C. The Reproduction of Aerobic Bacteria. (With Plates IV—VII) . . . . .	369
FERGUSON, MARGARET. The Diets of Labouring Class Families during the course of the War . . . . .	409
KAUNTZE, W. H. A Polyvalent Vaccine in the Treatment of Bacillary Dysentery in East Africa . . . . .	417
SAVAGE, WILLIAM G. Cats and Human Diphtheria . . . . .	448
CRAMER, W. and GYE, W. E. A Note on "Defence Rupture" and the Action of Electrolytes . . . . .	463
NANKIVELL, A. T. and STANLEY, J. M. The Contamination of Oysters. (With 1 Chart) . . . . .	465
INDEX OF AUTHORS . . . . .	473
INDEX OF SUBJECTS . . . . .	474

SOME FACTORS INFLUENCING THE ACTIONS OF DYES  
AND ALLIED COMPOUNDS ON BACTERIA<sup>1</sup>.

BY G. S. GRAHAM-SMITH, M.D.

*(From the Medical School, University of Cambridge.)*

(With Plate I and six charts.)

THE experiments recorded in this paper were undertaken in order to ascertain the effects of certain dyes and allied organic compounds on three selected species of bacteria, *Staphylococcus aureus*, *B. coli* and *B. pyocyaneus*. These experiments show that in cultures the results are influenced by many factors, and appear to suggest that the varying results of the use of these compounds in the treatment of wounds may be ascribed to some extent to the different conditions prevailing in each case.

The chief organic substances examined were diaminotrimethylacridinium chloride, termed for the sake of brevity, Homoflavine, Quinone and Crystal Violet. Homoflavine is more easily prepared on a technical scale than the ordinary acriflavine, with which it is homologous, being made from the acridine produced from metatoluylenediamine by combination with a methyl ester; both it and Crystal Violet have been produced on a manufacturing scale by Messrs Levinstein, Ltd, of Manchester, and pure material provided by this firm has been used in the present investigation. The other organic compounds named in the table on p. 4 have been carefully purified by Professor W. J. Pope; none of the dye stuffs comprised in this list were double compounds with metallic salts and their compositions are stated in Green's *Organic Colouring Matters*.

During the last few years many investigations on the bactericidal effects of dyes have been undertaken. Browning, Gulbransen, Kennaway and Thornton (1917), Dakin and Dunham (1917), and Nicholls (1917), employed 0·7 % peptone, Fleming (1917), Hewlett (1917), Morgan (1918), Taylor (1917) and Wright (1917) used "broth," Drummond and McNee (1917) "glucose broth," Morgan (1918) serum broth, Churchman (1912, 1913), Churchman and Michael (1912), Krumwiede and Pratt (1914) and Teague (1918) "ordinary agar" and Browning and Gilmour (1914) "peptone water agar." Presumably all these media contained the usual quantity of peptone. The use of peptone<sup>2</sup>

<sup>1</sup> A Report to the Medical Research Committee, April 25, 1918.

<sup>2</sup> Browning, Gulbransen and Thornton (1917) have shown that "for some unascertained reason the bacterial potency of flavine for staphylococcus in dilute peptone water shows considerable variations in an extended series of experiments."



has been avoided in these experiments, and consequently the results of these workers are not strictly comparable with those quoted in this paper.

Browning, Gulbransen, Kennaway and Thornton (1917), Fleming (1917) and Morgan (1918) used ox serum, Dakin and Dunham (1917) horse serum, and horse serum together with muscle extract, and Hewlett (1917) human serum. Blood was employed by Dakin and Dunham (1917), Fleming (1917) and Morgan (1918), pus by Fleming (1917) and Hewlett (1917), and milk by Hewlett (1917). None of these fluids were employed in the present experiments.

Varying results in preliminary experiments made it evident that to obtain comparable results media of simple and constant composition would be required, and that an arbitrary time limit would have to be adopted. As a fluid medium neutral meat extract and as a solid neutral meat extract agar were chosen and the actions of the compounds on the three species of organisms growing in the former and on the surface of the latter compared.

The meat extract was prepared from bullock's heart muscle after removing the fat, fascia, etc. To each 100 grms. of minced meat 250 c.c. of water were added, the fluid slowly boiled for one hour, filtered through filter paper and sterilised in the autoclave. The clear extract so obtained requires 0.08 c.c. of N/10 soda to render it neutral to neutral red.

On this medium all the organisms mentioned grow very well.

Tubes containing 1 c.c. of meat extract, 0.08 c.c. of N/10 soda, 3.5 c.c. of distilled water were prepared and sterilised by boiling. When cool 0.5 c.c. of a solution of the compound in distilled water and lastly a drop of an emulsion in sterile distilled water of the organism from a 24 hour old agar culture grown at 37° C. were added, and the culture incubated at 37° C. Care was taken to make emulsions as uniform in numbers of bacteria as possible, and on many occasions the organisms present in the cultures at the beginning of the experiment were estimated by means of plate cultures. The numbers in most of the experiments varied between 4,000 and 2,000 organisms per drop. The naked eye results were noted after 24 and 48 hours' incubation and to confirm them subcultures were sown on agar plates with a standard loop (0.01 c.c.).

In order to compare the results when the organisms were grown on the surface of agar 1 c.c. of meat extract, 2 c.c. of melted agar (2 % in distilled water), 0.08 c.c. of N/10 soda and 1.5 c.c. of distilled water were placed in tubes and sterilised by boiling. When cooled to 60° C. 0.5 c.c. of a solution of the compound was added. After thorough mixing the contents of the tube were poured into Petri dishes<sup>1</sup> and allowed to set. With the aid of a platinum loop three streaks of strong emulsions of the three organisms in distilled water

<sup>1</sup> Small Petri dishes divided according to the method devised by Churchman (1912) were employed in order to reduce the quantity of medium used and to facilitate comparison. Instead of metal divisions cardboard strips, cemented to the bottoms and sides of the dishes with water agar, were utilised.

were made across the surface of the medium. The cultures were incubated at 37° C. and the results were noted after 24 and 48 hours' incubation.

Table I (p. 4) gives the results of these experiments after 48 hours' incubation, a + indicating visible growth in the fluid medium or growth on the agar though only evidenced by the presence of a single colony, and a 0 the failure of the organism to grow.

In the agar series it frequently happened that though no growth was visible after 24 hours' incubation a few or even numerous colonies were found on examining the plate after 48 hours' incubation. Consequently the results obtained after 24 hours' incubation differ considerably from those obtained after 48 hours' incubation (Plate I, figs. 1-20). Since *in the agar series* further incubation seldom produces any change, the time limit of 48 hours was chosen, and unless otherwise stated the results recorded indicate the findings under these conditions.

*In the fluid medium* good growth may occur on subsequent days in tubes which show no evidence of growth in 2 days.

Table I, in which the results are tabulated in the order of the action of the compounds on staphylococci growing on the surface of neutral agar, shows that (1) under these conditions the effects of the compounds are not strictly correlated to their chemical relationship, (2) the substances most toxic to staphylococci have little toxicity to *B. coli* or *B. pyocyaneus*, and (3) of the three organisms *B. pyocyaneus* is the most resistant.

The second part of the table gives the action of the compounds on the organisms growing in meat extract, and shows that (1) under these conditions different values are obtained for many of the compounds, (2) a different order in efficiency is found, and (3) some of the compounds exhibit marked toxicity to *B. coli* and to a lesser degree to *B. pyocyaneus*.

The differences between the tables are especially noteworthy since agar has no nutritive value and the quantity of nutrient material (meat extract) is the same in both series.

Since Crystal Violet exhibits the greatest toxicity towards staphylococci, Quinone the greatest toxicity towards *B. coli* and *B. pyocyaneus*, when growing on agar, and homoflavine the greatest toxicity towards these organisms in meat extract, these three compounds were selected for further investigation.

### Homoflavine.

From Table I it will be seen that on agar the toxicity of homoflavine towards staphylococci is somewhat greater than that of methylhomoflavine or methylhomoacridine, while the toxicity of these three compounds towards *B. coli* and *B. pyocyaneus* is similar. In meat extract the toxicity of all three towards staphylococci is similar, but homoflavine and methylhomoflavine are more toxic to *B. coli* and to *B. pyocyaneus* than methylhomoacridine.



Table I.

Showing the least concentrations of the following compounds which cause inhibition of growth for 48 hours at 37° C.

	Neutral agar			Neutral meat extract		
	Staphylo- cocci	<i>B. coli</i>	<i>B. pyo- cyaneus</i>	Staphylo- cocci	<i>B. coli</i>	<i>B. pyo- cyaneus</i>
1. Crystal violet	1: 3,250,000	1: 10,000	1: 10,000	1: 10,000,000	—	—
2. Brilliant red Rhoduline	1: 1,000,000	1: 1,000	1: 1,000	1: 500,000	1: 10,000	1: 1,000
3. Irisamine	1: 900,000	1: 1,000	1: 1,000	1: 1,100,000	1: 1,000	1: 1,000
4. Metaphenylene Blue B	1: 200,000	1: 1,000	1: 1,000	1: 900,000	1: 10,000	1: 10,000
5. Pyronine G	1: 150,000	1: 1,000	1: 1,000	1: 200,000	1: 20,000	1: 10,000
6. Pararosanine hydro- chloride	1: 70,000	1: 1,000	1: 1,000	1: 60,000	1: 1,000	1: 1,000
7. Quinone	1: 70,000	1: 95,000	1: 30,000	1: 60,000	1: 100,000	1: 10,000
8. Hydroquinone	1: 60,000	1: 40,000	1: 10,000	1: 20,000	1: 20,000	1: 10,000
9. Safranine S	1: 50,000	1: 1,000	1: 1,000	1: 70,000	1: 20,000	1: 1,000
10. Acridine red	1: 50,000	1: 10,000	1: 1,000	1: 30,000	1: 1,000	1: 1,000
11. Isonitrophenol	1: 40,000	1: 20,000	1: 10,000	1: 20,000	1: 20,000	1: 10,000
12. Chrysoïdine T	1: 20,000	1: 10,000	1: 1,000	1: 30,000	1: 10,000	1: 1,000
13. Homoflavine	1: 14,000	1: 9,000	1: 1,000	1: 300,000	1: 250,000	1: 30,000
14. Methylhomoflavine	1: 10,000	1: 10,000	1: 1,000	1: 300,000	1: 250,000	1: 30,000
15. Methylhomoacridine	1: 10,000	1: 10,000	1: 1,000	1: 300,000	1: 200,000	1: 10,000
16. Toluidine blue acid	1: 10,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
17. Congo red acid	1: 10,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
18. Phosphine R	1: 10,000	1: 1,000	1: 1,000	—	—	—
19. Rhodamine B	1: 10,000	1: 1,000	1: 1,000	1: 10,000	1: 1,000	1: 1,000
20. Rhodamine S	1: 10,000	1: 1,000	1: 1,000	1: 70,000	1: 1,000	1: 1,000
21. Erythrosine	1: 10,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
22. Alizarine red	1: 1,000	1: 1,000	1: 1,000	1: 10,000	1: 1,000	1: 1,000
23. Orange G	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
24. Night blue basic	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
25. Diamine blue 3 B	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
26. Crystal scarlet 6 R	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
27. Benzo blue 6 B neutral	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
28. Diaminogene blue G	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
29. Quinoline yellow S	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
30. Acid violet 6 B	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
31. Magenta S	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
32. Thioflavine S	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
33. Tartrazine	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
34. Trypan red	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
35. Trypan blue	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
36. Auramine O	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
37. Patent blue	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
38. Naphthol green	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000

Concentrations greater than 1: 1,000 were not tested, and the sign 1: 1,000 indicates that good growth took place at a concentration of 1: 1,000.

It will be noticed that the influence on staphylococci of Nos. 2 and 8 is much greater on agar than in meat extract, of numbers 10, 11, 16, 17, and 21 slightly greater, while the influence of numbers 1, 4, 13, 14, 15, and 20 is much greater in meat extract than on agar, and of numbers 3, 5, 9, 12, and 22 slightly greater. The influence of numbers 6, 7, and 19, and probably of numbers 23–38, is similar in both media.



*The effects of varying the proportion of nutrient material and the reaction of the medium.*

In order to ascertain the effects of varying the proportion of the nutrient material and the reaction of the medium several series of experiments were carried out both in meat extract and on meat extract agar. In each case the total quantity of the medium employed was 5 c.c., but the quantity of nutrient material (meat extract) varied between 0.5 c.c. and 2 c.c. For example at neutrality to neutral red the meat extract series was made up in the following manner.

	Meat extract	N/10 soda	Distilled water	Solution of homoflavine
A	0.5*	0.04	3.96	0.5
B	1.0	0.08	3.42	0.5
C	1.5	0.12	2.88	0.5
D	2.0	0.16	2.34	0.5

\* In this and other tables describing the composition of media the figures indicate the quantities of ingredients used in c.c.

And the agar series as follows.

	Meat extract	Agar (2%)	N/10 soda	Distilled water	Solution of homoflavine
A	0.5	2.0	0.04	1.96	0.5
B	1.0	2.0	0.08	1.42	0.5
C	1.5	2.0	0.12	0.88	0.5
D	2.0	2.0	0.16	0.34	0.5

In order to vary the reaction on the alkaline side additional quantities of N/10 soda (0.1, 0.2, 0.3, 0.4, 0.5 c.c.) were added beyond the neutral point with a corresponding diminution in the amount of water. On the acid side either no addition was made, or in some cases N/10 hydrochloric acid (0.05, 0.1 and 0.15 c.c.) was added with a corresponding diminution in the amount of water.

Thus in the experiments with the fluid medium containing 1 c.c. of meat extract the whole series was made up in the following manner.

	Meat extract	N/10 soda	N/10 HCl	Distilled water	Solution of homoflavine
I	1.0	—	0.1	3.4	0.5
II	1.0	—	0.05	3.45	0.5
III	1.0	—	—	3.5	0.5
IV	1.0	0.08	—	3.42	0.5
V	1.0	0.18	—	3.32	0.5
VI	1.0	0.28	—	3.22	0.5
VII	1.0	0.38	—	3.12	0.5
VIII	1.0	0.48	—	3.02	0.5
IX	1.0	0.58	—	2.92	0.5

The concentration of homoflavine causing complete inhibition of growth during 48 hours' incubation at 37° C. is given in Table II and Charts I and II.

Table II.

Showing the concentration of homoflavine required to cause complete inhibition of growth after 48 hours' incubation at 37° C. on agar and in meat extract of different reactions and containing different quantities of nutrient material.

Quantity of acid or alkali added	Quantity of meat extract	Agar			Meat extract		
		<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
0.1 N/10 HCl	1.0	1: 1,500	1: 4,000	1: 500	—	1: 200,000	1: 30,000
0.05 „	1.0	1: 1,500	1: 8,000	1: 500	1: 150,000	1: 250,000	1: 20,000
No addition	0.5	1: 9,000	1: 18,000	1: 500	1: 300,000	1: 500,000	1: 40,000
	1.0	1: 4,500	1: 10,000	1: 500	1: 150,000	1: 300,000	1: 20,000
	1.5	1: 3,500	1: 9,000	1: 500	1: 100,000	1: 200,000	1: 17,000
	2.0	1: 2,000	1: 6,000	1: 500	1: 100,000	1: 150,000	1: 13,000
0.08 N/10 soda per c.c. of meat extract*	0.5	1: 11,000	1: 20,000	—			
	1.0	1: 9,000	1: 14,000	—			
	1.5	1: 8,000	1: 11,000	—			
	2.0	1: 7,000	1: 10,000	—			
0.18 N/10 soda	0.5	1: 35,000	1: 30,000	—			
	1.0	1: 12,000	1: 17,000	—			
	1.5	1: 8,000	1: 15,000	—			
	2.0	1: 7,000	1: 10,000	—			
0.28 „	0.5	1: 60,000	1: 75,000	—			
	1.0	1: 20,000	1: 20,000	—			
	1.5	1: 9,000	1: 15,000	—			
	2.0	1: 8,000	1: 12,000	—			
0.38 „	0.5	1: 100,000	1: 160,000	1: 1,500	1: 1,800,000	1: 1,800,000	1: 110,000
	1.0	1: 45,000	1: 40,000	1: 1,500	1: 1,600,000	1: 1,400,000	1: 70,000
	1.5	1: 11,000	1: 16,000	1: 1,500	1: 1,100,000	1: 1,100,000	1: 50,000
	2.0	1: 10,000	1: 15,000	1: 1,500	1: 1,000,000	1: 800,000	1: 40,000
0.48 „	0.5	1: 290,000	1: 300,000	—			
	1.0	1: 90,000	1: 90,000	—			
	1.5	1: 15,000	1: 21,000	—			
	2.0	1: 14,000	1: 21,000	—			
0.58 „	0.5	1: 300,000	1: 320,000	1: 1,500	1: 2,000,000	1: 2,000,000	1: 130,000
	1.0	1: 145,000	1: 160,000	1: 1,500	1: 1,800,000	1: 1,800,000	1: 110,000
	1.5	1: 18,000	1: 23,000	1: 1,500	1: 1,400,000	1: 1,600,000	1: 100,000
	2.0	1: 17,000	1: 26,000	1: 1,500	1: 1,200,000	1: 1,400,000	1: 90,000

\* 1 c.c. of meat extract requires 0.08 c.c. N/10 soda to neutralise it to neutral red. In cultures containing 0.5, 1.5 and 2.0 c.c. of meat extract proportionate quantities of N/10 soda were added. In the alkaline series 0.1, 0.2, 0.3, 0.4, 0.5 c.c. of N/10 soda were added beyond the quantities sufficient to bring the reaction to neutrality. For the sake of lucidity these figures have been omitted in Table II and Charts I, II and III, only the quantities added to cultures containing 1 c.c. being quoted.

It will be seen from Table II and Charts I and II that under all conditions the smaller the amount of nutrient material present the greater the efficiency of the homoflavine solution, and that in regard to *B. coli* and staphylococci its efficiency is greatly increased by the addition of small quantities of N/10 soda. On agar with the larger quantities of nutrient material the successive increments of soda have less effect than with the smaller quantities. In meat extract cultures this phenomenon is much less marked.

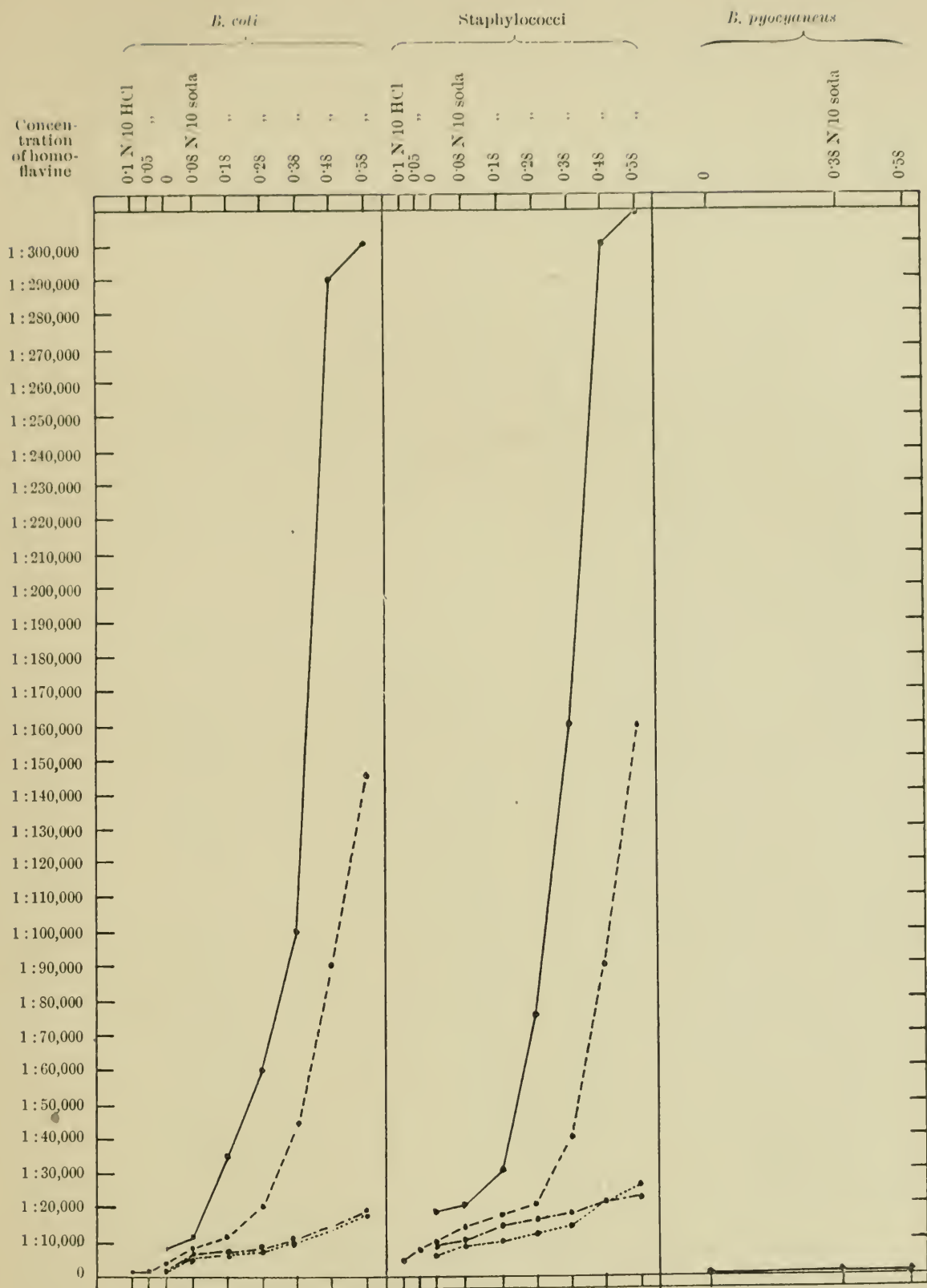


CHART I. Showing the influence of the quantity of nutrient material and change of reaction on the concentration of homoflavine necessary to cause inhibition of growth on agar during 48 hours' incubation at 37° C.

— 0.5 c.c. meat extract  
--- 1.0 c.c. " "  
- . - 1.5 c.c. meat extract  
..... 2.0 c.c. " "

## Action of Dyes on Bacteria

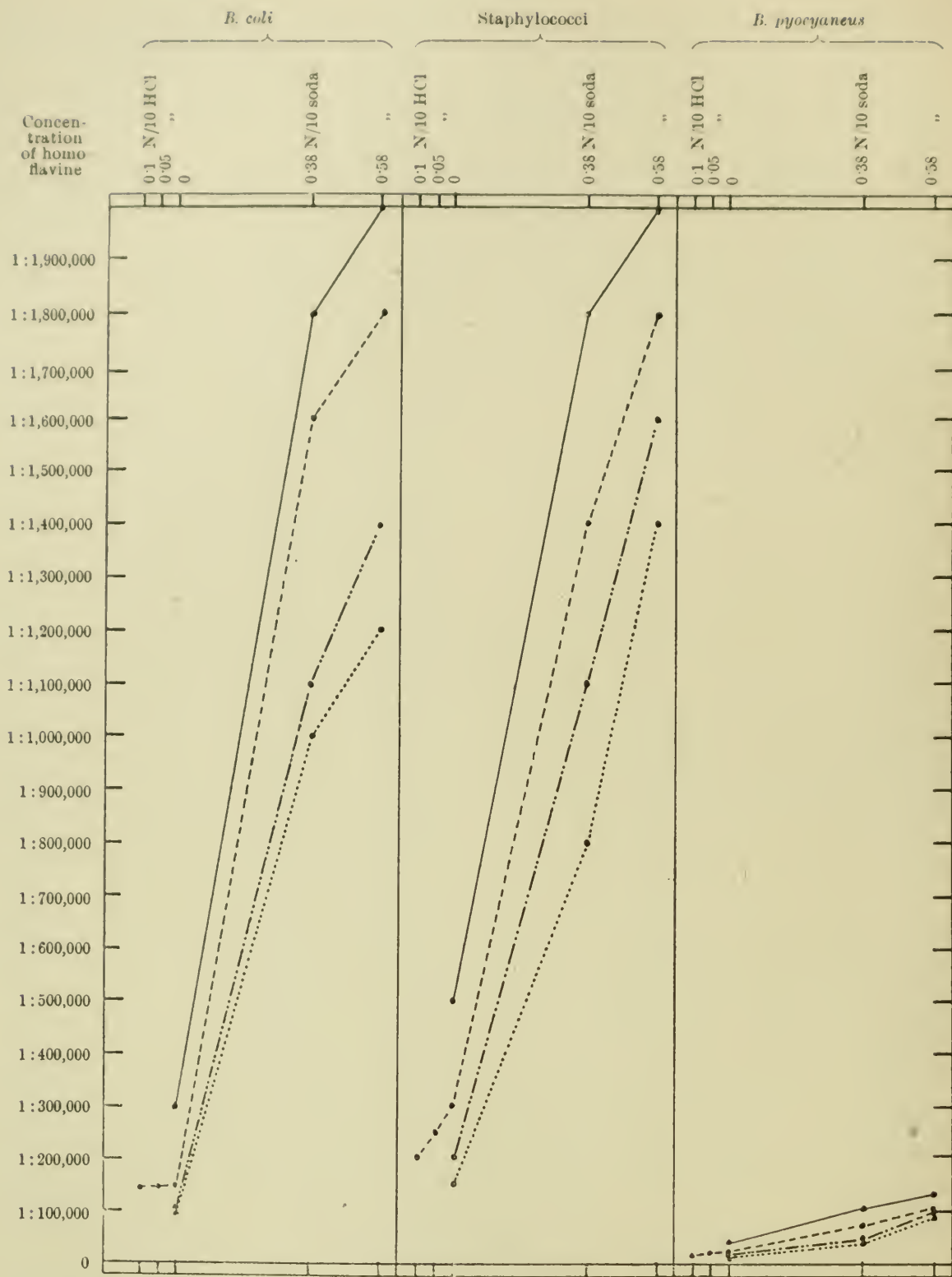


CHART II. Showing the influence of the quantity of nutrient material and change of reaction on the concentration of homoflavine necessary to cause inhibition of growth in meat extract during 48 hours' incubation at 37° C.

—— 0.5 c.c. meat extract

— · — · 1.5 c.c. meat extract

----- 1.0 c.c. ,, ,,

..... 2.0 c.c. ,, ,,



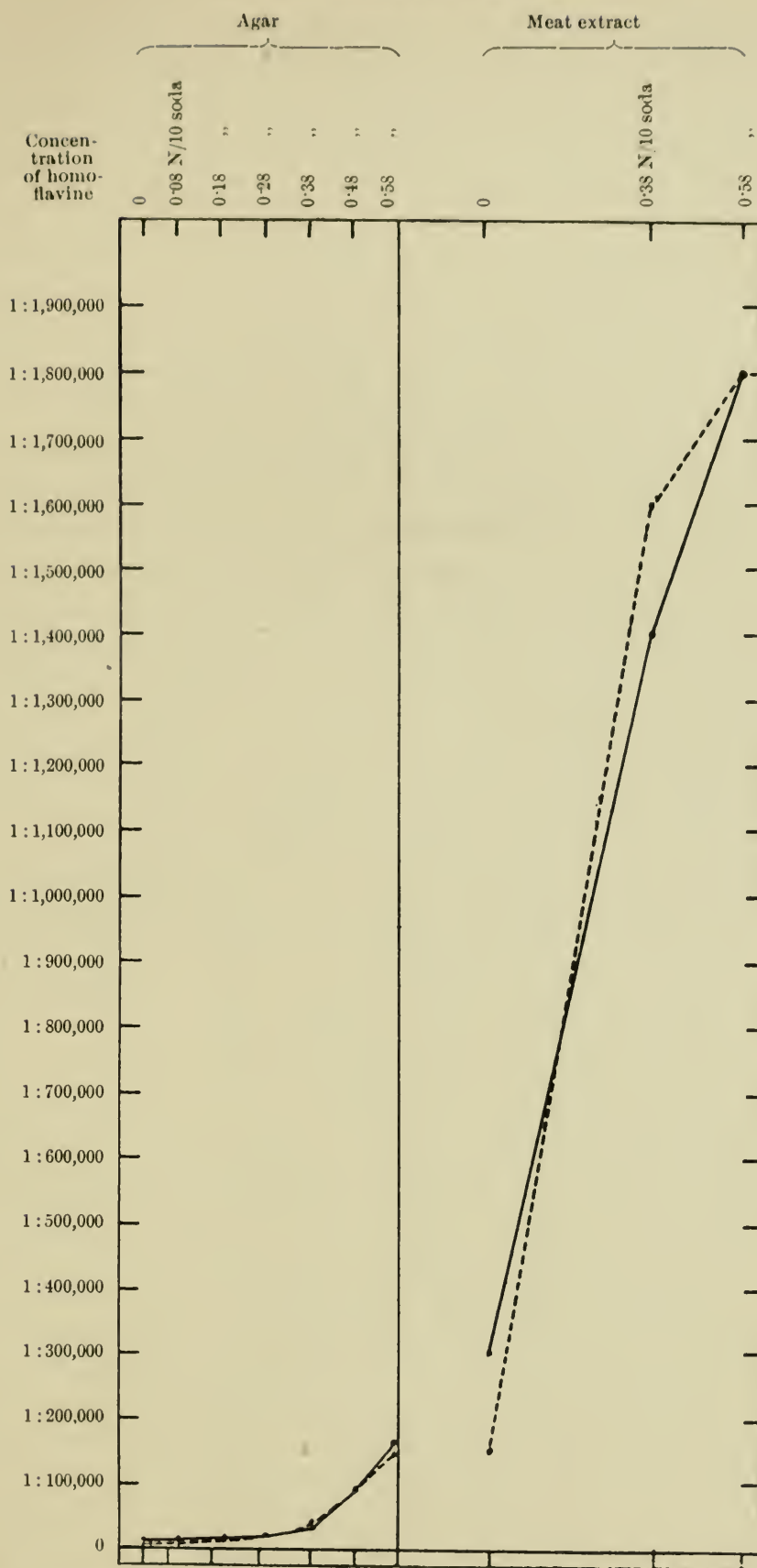


CHART III. Illustrating the differences in concentrations of homoflavine necessary to inhibit the growth of staphylococci and *B. coli* during 48 hours' incubation at 37° C. when growing on agar and in meat extract, when each tube contains 1 c.c. of nutrient material.

--- *B. coli*

— staphylococci

It will be noticed that the curves for *B. coli* and staphylococci are very similar. In this connection it is of interest to note that these organisms multiply at much the same rate on meat extract (without the addition of homoflavine) as shown by counting the colonies on agar subcultures made at different periods of growth. On the other hand *B. pyocyaneus* multiplies much more rapidly.

The action of homoflavine on *B. pyocyaneus* on agar is very little influenced by the addition of soda, and not very greatly influenced in the meat extract.

Chart III has been constructed to bring out more clearly the remarkable difference between the actions of homoflavine in agar and in meat extract when acting on *B. coli* and staphylococci.

In order to ascertain whether the striking differences between the inhibiting concentrations on agar and in meat extract were due to the prevalence of aerobic conditions in the agar plates and partial anaerobic conditions in the neutral meat extract tubes, cultures were made in aerated meat extract and boiled meat extract under paraffin. The results were almost identical. The growth of the staphylococci was inhibited in each series at a concentration of 1:550,000, of *B. coli* at 1:250,000 and of *B. pyocyaneus* of about 1:40,000.

A series of experiments were carried out to ascertain the influence of reaction on the growth of these organisms on the media described with varying quantities of meat extract, replacing the homoflavine solution with distilled water. The results are given in the following table.

	Agar			Meat extract		
	<i>B. coli</i>	Staphylo- cocci	<i>B. pyo- cyaneus</i>	<i>B. coli</i>	Staphylo- cocci	<i>B. pyo- cyaneus</i>
	2 c.c. meat extract	2 c.c. meat extract	2 c.c. meat extract	1 c.c. meat extract	1 c.c. meat extract	1 c.c. meat extract
	1 " "	1 " "	1 " "			
	0.5 " "	0.5 " "	0.5 " "			
N/10 soda						
2.5 c.c.	- - -	- - -	- - -	-	-	-
2.25	- - -	+ - -	- - -			
2.0	+ - -	+ + -	+ + +			
1.9	+ - -	+ + -	+ + +			
1.8	+ - -	+ + -	+ + +			
1.7	+ + -	+ + -	+ + +	-	+	+
1.6	+ + +	+ + +	+ + +	-	+	+
1.5	+ + +	+ + +	+ + +	-	+	+
1.4	+ + +	+ + +	+ + +	+	+	+
N/10 HCl						
0.1 c.c.	+ + +	+ + +	+ + +	+	+	+
0.125	+ + +	+ + +	+ + +	+	+	+
0.150	+ + -	+ + -	+ + -	+	-	+
0.175	+ + -	+ + -	+ - -	-	-	-
0.2	+ - -	+ - -	+ - -			
0.3	- - -	- - -	- - -			

+ = growth after 48 hours' incubation. - = no growth.



This table shows that the quantity of acid or alkali which is necessary to add in order to inhibit growth depends to some extent on the quantity of nutrient material present. While on the alkaline side the range is considerable, the range on the acid side is very small, but it may be increased to some extent by increasing the concentration of meat extract. With 2.5 c.c. of meat extract growth of staphylococci occurs in the fluid medium when 0.3 c.c. of N/10 HCl is added, and with 5 c.c. of meat extract when 0.8 c.c. of N/10 HCl is present.

Such small quantities of N/10 soda as greatly influence the action of homoflavine do not of themselves appear to have any influence on the growths, when these are compared with neutral controls.

In the case of *B. coli* and *B. pyocyaneus* the organisms are very short, rounded and almost coccus-like when growing on the acid medium, and very long, thin and irregular when growing on the higher concentrations of soda.

*The effect of varying the proportion of agar.*

To determine whether the quantity of agar employed has any influence on the concentration of homoflavine necessary to inhibit the growth of *B. coli* and staphylococci, experiments with media of the following composition were carried out.

	Meat extract	Agar (2%)	N/10 soda	Distilled water	Solution of homoflavine
A	1.0	1.0	0.08	2.42	0.5
B	1.0	2.0	0.08	1.42	0.5
C	1.0	3.0	0.08	0.42	0.5

After 48 hours' incubation at 37° C. it was seen that different concentrations of homoflavine were necessary to inhibit growth in these three media.

	Growth inhibited at a concentration of	
	<i>B. coli</i>	Staphylococci
A	1 : 14,000	1 : 30,000
B	1 : 9,000	1 : 14,000
C	1 : 7,000	1 : 9,000

Homoflavine does not seem to enter into strong combination with the agar, for if divided plates are made according to Churchman's (1912) method, having a solution of homoflavine mixed with the agar on one side and not on the other, and the partition walls are removed immediately after the agar has set, and subsequently emulsions of organisms are stroked across the medium at right angles to the dividing line, the plain medium becomes coloured for a short distance beyond the dividing line and colonies at a considerable distance beyond the line take up the stain and become yellow.

The diffusion of homoflavine and its consequences can be well illustrated in another manner. Two parallel streaks of plain agar (2 %), one broad and the other narrow, are made on the bottom of a Petri dish and allowed to set, and then medium of the composition of B in the experiment just quoted is poured into the dish so as to cover the streaks and allowed to set. Subsequently emulsions of *B. coli* and staphylococci are stroked across the plate at right angles to the agar streaks. At a concentration of 1 : 9,000 a few colonies of

*B. coli* grow immediately over the streaks, but not elsewhere, though when the concentration decreases to 1:17,000 colonies appear wherever the emulsion was spread, but most thickly over the streaks. Similarly colonies of staphylococci grow over the broad streak only at a concentration of 1:10,000, over both streaks at 1:14,000 and wherever the emulsion was spread at a concentration of 1:14,000 and wherever the emulsion was spread at a concentration of 1:20,000, showing that the homoflavine diffuses into the plain agar streaks, and consequently so lowers the concentration over them that the organisms can grow though inhibited elsewhere (Plate I, fig. 21).

Morphologically the *B. coli* from the colonies growing over the streaks at a concentration of 1:9,000 are very long and irregular, but those growing in the same situation at a concentration of 1:17,000 are much shorter and more normal in appearance. The change in morphology from very long to short normal forms may be traced in the divided plates just mentioned when passing from the extremity of the stroke of emulsion on the side containing the homoflavine to the opposite extremity.

Strong concentrations of homoflavine (1:1,000) seem to cause the agar to set very firmly and to lessen the exudation of water from it.

*The effects of salt and of peptone on agar cultures.*

In order to ascertain the influence of the presence of 0.75 % salt and 1 % peptone on agar cultures the following series A, B, C, D of experiments were carried out.

	The members of each series contained				There were added in						
	Meat extract	Agar	N/10 HCl	N/10 soda	Series A	Series B		Series C		Series D	
					Dis-tilled water	15 % salt	Dis-tilled water	20 % peptone	Dis-tilled water	20 % pep- tone in 15 % salt	Dis-tilled water
1.	1.0	2.0	0.4	—	1.6	0.25	1.35	0.25	1.35	0.25	1.35
2.	1.0	2.0	0.3	—	1.7	0.25	1.45	0.25	1.45	0.25	1.45
3.	1.0	2.0	0.2	—	1.8	0.25	1.55	0.25	1.55	0.25	1.55
4.	1.0	2.0	0.1	—	1.9	0.25	1.65	0.25	1.65	0.25	1.65
5.	1.0	2.0	—	—	2.0	0.25	1.75	0.25	1.75	0.25	1.75
6.	1.0	2.0	—	1.0	1.0	0.25	0.5	0.25	0.5	0.25	0.5
7.	1.0	2.0	—	1.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25
8.	1.0	2.0	—	1.75	0.25	0.25	0.0	0.25	0.0	0.25	0.0
9.	1.0	2.0	—	0.2 (N)	1.8	0.25	1.55	0.25	1.55	0.25	1.55
10.	1.0	2.0	—	0.25 (N)	1.75	0.25	1.5	0.25	1.5	0.25	1.5

After 48 hours' incubation at 37° C. the results were as follows:

	<i>B. coli</i> Series				Staphylococci Series				<i>B. pyocyaneus</i> Series			
	A	B	C	D	A	C	B	D	A	B	C	D
1.	0	0	0	+	0	0	0	×	0	0	0	0
2.	0	0	×	+	0	0	×	+	0	0	0	0
3.	×	+	+	+	×	+	+	+	0	×	+	+
4.	+	+	+	+	+	+	+	+	+	+	+	+
5.	+	+	+	+	+	+	+	+	+	+	+	+
6.	+	+	+	+	+	+	+	+	+	+	+	+
7.	+	+	+	+	+	+	+	+	+	+	+	+
8.	+	+	+	+	+	+	+	+	+	+	+	+
9.	+	×	0	0	+	+	0	×	+	+	0	0
10.	+	0	0	0	+	+	0	0	+	0	0	0

+ = good growth, × = slight growth, and 0 = no growth.



In the case of *B. coli* the presence of salt or peptone diminishes the range of growth on the alkaline side, and slightly increases it on the acid side, but the presence of both distinctly increases it on the acid side. In the case of staphylococcus the presence of salt has little effect, but peptone decreases the range on the alkaline side, and the presence of both increases the range on the acid side. In the case of *B. pyocyaneus* the range on the acid side is slightly increased and on the alkaline side distinctly decreased by both salt and peptone alone or combined.

*The action of homoflavine on cultures of various ages.*

Cultures were grown on meat extract 1 c.c., N/10 soda 0.08 c.c., water 3.5 c.c. for one, three and ten days respectively. At the expiration of these periods 0.5 c.c. of a solution of homoflavine was added, and the tubes returned to the incubator. After one, two and eight days' incubation a loopful from each tube was sown on agar, and the result recorded after two days at 37° C.

Concentration of homoflavine for <i>B. coli</i> and staphylococci	Cultures 24 hours old									
	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine			After 8 days' contact with homoflavine		
	<i>B. coli</i>	Staphylococci	Concentration for <i>B. pyocyaneus</i>	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 50,000	0	0	1: 550	0	0	0	0	few	0	0
1: 100,000	numerous	many	1: 750	0	many	0	0	numerous	0	0
1: 150,000	„	numerous	1: 1,000	0	„	0	0	„	0	0
1: 200,000	„	„	1: 2,000	numerous	„	0	few	„	0	0
1: 250,000	„	„	1: 10,000	„	numerous	0	numerous	„	0	numerous

Concentration for <i>B. coli</i> and staphylococci	Cultures three days old									
	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine			After 8 days' contact with homoflavine		
	<i>B. coli</i>	Staphylo- cocci	Concentration for <i>B.</i> <i>pyocyaneus</i>	<i>B. pyo- cyaneus</i>	<i>B. coli</i>	Staphylo- cocci	<i>B. pyo- cyaneus</i>	<i>B. coli</i>	Staphylo- cocci	<i>B. pyo- cyaneus</i>
1: 50,000	numerous	few	1: 550	0	0	0	0	0	0	0
1: 100,000	„	„	1: 750	0	few	0	0	numerous	0	0
1: 150,000	„	numerous	1: 1,000	few	„	0	0	„	0	0
1: 200,000	„	„	1: 2,000	„	„	0	0	„	0	0
1: 250,000	„	„	1: 10,000	many	many	few	few	„	0	0

Concentration for <i>B. coli</i> and staphylococci	Cultures 10 days old						
	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine		
	<i>B. coli</i>	Staphylococci	Concentration for <i>B. pyocyaneus</i>	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 50,000	few	0	1: 550	0	0	0	0
1: 100,000	numerous	0	1: 750	0	0	0	0
1: 150,000	„	0	1: 1,000	numerous	few	0	0
1: 200,000	„	few	1: 2,000	„	„	0	0
1: 250,000	„	„	1: 10,000	„	„	0	0

These experiments, which have not been repeated, seem to show that in cultures during the rapidly growing stages staphylococci are killed more readily than *B. coli* by the prolonged action of certain strengths of the homoflavine. In the case of *B. coli* after a period of inhibition multiplication may occur. The older cultures of all these organisms seem to be the most susceptible to the prolonged action of homoflavine.

If cultures on such a medium (the homoflavine being replaced by distilled water) be examined by means of dilutions in plate cultures it will be found that at the end of 24 hours' incubation great multiplication of the organisms has taken place, and that by the third day the growth in the case of *B. coli* and staphylococci has nearly reached its maximum. Subsequently multiplication ceases, and by the tenth day a great reduction in numbers has taken place. *B. pyocyaneus* reaches its maximum very early, and active multiplication has ceased by the third day.

It is evident from these experiments that the results obtained by adding solutions of homoflavine to actively growing or declining cultures are very different from those obtained in previous experiments in which relatively small numbers of organisms were added to media already containing homoflavine.

The experiments which have been quoted show that the action of homoflavine is very greatly influenced by the reaction of the medium, the quantity of nutrient substance, the presence of agar, and the age of the culture; in fact any alteration in the composition of the medium, or the proportion of the ingredients, affects the results obtained to a greater or less degree. (See also pp. 16, 18).

#### *Comparison of Acriflavine and Homoflavine.*

The effects of acriflavine and homoflavine in meat extract cultures have not been compared, but some comparative experiments in ox serum, sterilised by heating to 55° C., and in 0·7 % peptone water, were made.

In each tube were placed 0·8 c.c. of serum or 0·8 c.c. of 0·7 % peptone water, 0·1 c.c. of a solution of the dye and 0·1 c.c. of a dilution (staphylococci 1 : 20,000, *B. coli* 1 : 10,000) in 0·75 % salt solution of a 24 hours' peptone water culture of the organism. Fortunately the two dilutions contained almost the same number of organisms. Control tubes without the dye were sown at the same time.

The following table (p. 15) shows the results of this experiment, the figures indicating the numbers of organisms growing in cultures on agar made with one standard loopful (0·01 c.c.), after dilution if this seemed necessary.

The peptone solution was distinctly acid, 5 c.c. requiring 0·275 N/10 soda to neutralise it to neutral red, and the serum distinctly alkaline, 5 c.c. requiring 0·75 N/10 HCl to neutralise it.

These experiments show that the actions of the two dyes on staphylococci and *B. coli* respectively are very similar, but that while the staphylococci are



Staphylococci													
Cultures in 0.7% peptone													
Concentration of dye	Acridine					Homoflavine					Acridine		
	23	51	73 hrs.	5	23	51	73 hrs.	5	23	51	73 hrs.	5	73 hrs.
1: 50,000	0	0	0	-	0	0	0	-	0	0	0	-	-
1: 100,000	0	0	0	16	0	0	0	1	0	0	-	3	0
1: 125,000	0	0	0	-	0	0	0	-	0	-	14,672	-	0
1: 150,000	0	0	0	16	0	0	0	20	262	7,250	365,000	1	6
1: 175,000	0	0	0	-	0	0	0	-	750	-	-	-	1,056
1: 200,000	0	0	0	14	0	0	0	33	51,000	640,000	-	16	973,000
1: 250,000	89,000	5,128,000	-	-	0	0	0	-	982,000	1,932,000	-	-	1,148,000
Immediate													
Control 30	68	12,300,000	-	-	Control 32	225	2,120,000	-	-	-	-	-	-

<i>B. coli</i>													
Cultures in 0.7% peptone													
Concentration of dye	Acridine					Homoflavine					Acridine		
	23	51	73 hrs.	5	23	51	73 hrs.	5	23	51	73 hrs.	5	73 hrs.
1: 50,000	0	0	0	-	0	-	0	-	0	0	0	-	0
1: 100,000	0	0	0	2	15	5,500	17,760	0	0	0	0	0	0
1: 125,000	90,880	-	-	-	50	-	39,360	-	0	-	0	-	0
1: 150,000	220,000	402,000	-	1	104,800	-	148,000	1	0	0	0	0	0
1: 175,000	400,000	-	-	-	-	-	-	-	0	0	0	-	0
1: 200,000	843,200	-	-	4	302,000	-	1,180,000	1	0	0	0	0	0
1: 250,000	1,392,000	3,208,000	-	-	-	-	-	-	0	0	0	-	0
Immediate													
Control 31	48	4,048,000	10,016,000	-	-	-	-	-	Control 37	427	7,112,000	-	-

0 = no growth in subculture, - = subculture not made.

more susceptible when growing in peptone water the *B. coli* are more susceptible when growing in serum. In the lower concentrations the organisms in many cases at first seemed to decrease in numbers and then to increase.

### Quinone.

Several series of experiments, comparable with those made with homoflavine, were carried out with quinone.

#### *The effects of varying the reaction of the medium.*

To test the effect of alterations in the reaction of the medium experiments were made on agar and in meat extract. In the former series the agar was melted, and the soda and water added. It was then cooled to 45° C., the solution of quinone added, the contents of the tube thoroughly mixed and the medium poured into plates and allowed to set.

	Meat extract	N/10 soda	N/10 HCl	Agar	Water	Solution of quinone
1	1.0	—	0.15	2.0	1.35	0.5
2.	1.0	—	0.1	2.0	1.4	0.5
3.	1.0	—	0.05	2.0	1.45	0.5
4.	1.0	—	—	2.0	1.5	0.5
5.	1.0	0.08	—	2.0	1.42	0.5
6.	1.0	0.18	—	2.0	1.32	0.5
7.	1.0	0.28	—	2.0	1.22	0.5
8.	1.0	0.38	—	2.0	1.12	0.5
9.	1.0	0.48	—	2.0	1.02	0.5
10.	1.0	0.58	—	2.0	0.92	0.5

The meat extract series was similar in all respects except that 2.0 c.c. of water were substituted for the 2.0 c.c. of agar. The solution of quinone was added when the medium had cooled after sterilisation.

The results are given in the following table, which shows the concentration of quinone necessary in order to completely inhibit growth for 48 hours when the agar and meat extract contain 1 c.c. of nutrient material.

	Agar			Meat extract		
	<i>B. coli</i>	Staphylo- cocci	<i>B. pyo- cyaneus</i>	<i>B. coli</i>	Staphylo- cocci	<i>B. pyo- cyaneus</i>
N/10 HCl						
1. = 0.15	1 : 100,000	1 : 45,000	1 : 30,000	—	—	—
2. = 0.1	1 : 90,000	1 : 40,000	1 : 20,000	—	1 : 120,000	1 : 55,000
3. = 0.05	1 : 70,000	1 : 50,000	1 : 25,000	1 : 120,000	1 : 60,000	1 : 70,000
4. = 0	1 : 90,000	1 : 70,000	1 : 25,000	1 : 90,000	1 : 50,000	1 : 7,500
N/10 soda						
5. = 0.08	1 : 95,000	1 : 70,000	1 : 30,000	1 : 110,000	1 : 60,000	1 : 10,000
6. = 0.18	1 : 90,000	1 : 55,000	1 : 30,000	1 : 90,000	1 : 40,000	1 : 15,000
7. = 0.28	1 : 75,000	1 : 45,000	1 : 27,000	1 : 40,000	1 : 30,000	1 : 15,000
8. = 0.38	1 : 60,000	1 : 30,000	1 : 25,000	1 : 25,000	1 : 20,000	1 : 12,500
9. = 0.48	1 : 50,000	1 : 20,000	1 : 20,000	1 : 17,500	1 : 25,000	1 : 10,000
10. = 0.58	1 : 45,000	1 : 15,000	1 : 15,000	1 : 10,000	1 : 25,000	1 : 7,500

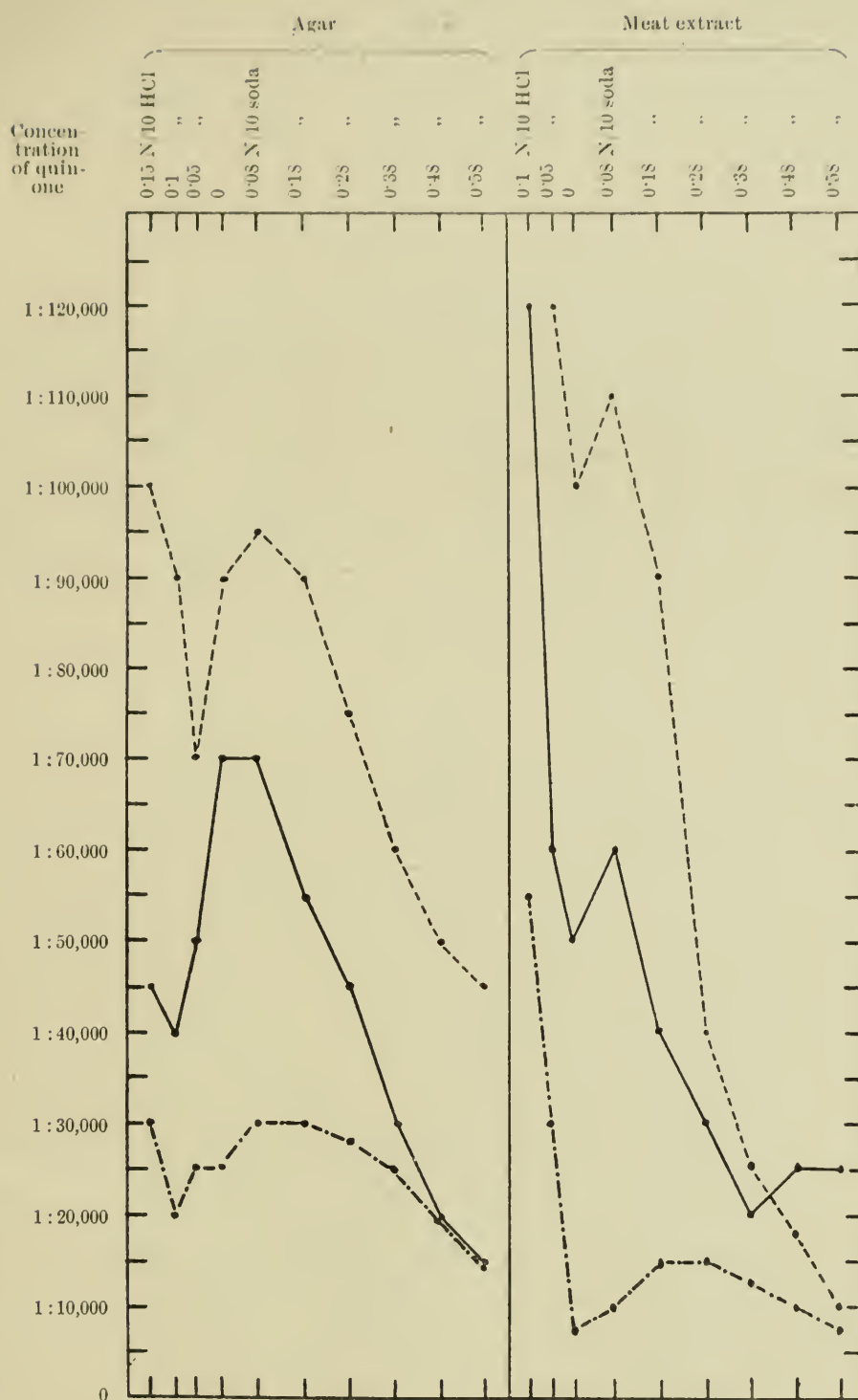


CHART IV. Showing the influence of change of reaction on the concentration of quinone necessary to cause inhibition of growth during 48 hours at 37°C. on agar and in meat extract.

----- *B. coli*

-.-.-.- *B. pyocyaneus*

— staphylococci

It will be seen by comparing Charts I, II, III, and IV that while the efficiency of homoflavine increases with the increase of alkalinity the efficiency of quinone diminishes. While homoflavine is most active in an alkaline solution, quinone is most active at or near neutrality to neutral red. Further there is not so marked a difference between the concentrations of quinone necessary to produce inhibition in agar and in meat extract as there is in the case of homoflavine.

*The effect of varying the proportion of nutrient material.*

The efficiency of quinone decreases as the concentration of nutrient material increases as shown by the following experiment on agar.

	Composition of medium				Solution of quinone	Concentration of quinone required to inhibit		
	Meat extract	Agar	N/10 soda	Water		<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1.	0.5	2.0	0.04	2.0	0.5	1 : 150,000	1 : 110,000	1 : 15,000
2.	1.0	2.0	0.08	1.5	0.5	1 : 85,000	1 : 65,000	1 : 12,000
3.	1.5	2.0	0.12	1.4	0.5	1 : 60,000	1 : 35,000	1 : 10,000
4.	2.0	2.0	0.16	1.3	0.5	1 : 50,000	1 : 25,000	1 : 8,000

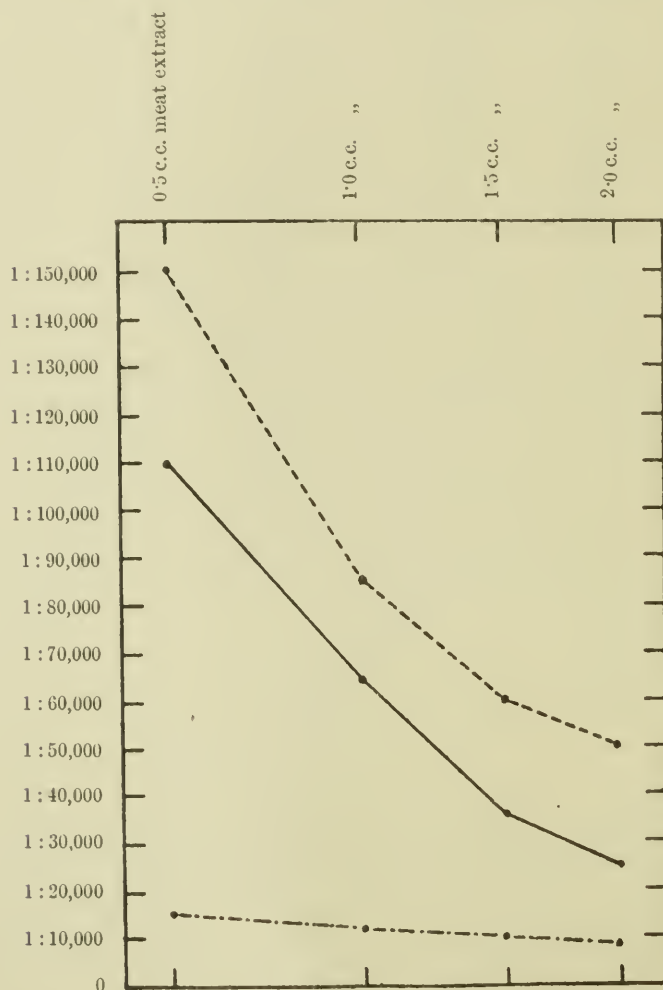


CHART V. Showing the concentrations of quinone necessary to cause inhibition of growth on agar during 48 hours at 37° C. in the presence of different quantities of nutrient material.

----- *B. coli*                      ——— *staphylococci*  
 -.-.-.- *B. pyocyaneus*



*The effect of varying the proportion of agar.*

The efficiency of quinone is only slightly influenced by the quantity of agar present.

	Composition of medium				Solution of quinone	Concentration of quinone required to inhibit the growth of staphylococci
	Meat extract	Agar	N/10 soda	Water		
1.	1.0	1.0	0.08	2.42	0.5	1: 60,000
2.	1.0	2.0	0.08	1.42	0.5	1: 70,000
3.	1.0	3.0	0.08	0.42	0.5	1: 80,000

*The effect of heat on the action of quinone.*

The action of quinone, unlike the action of homoflavine, is greatly influenced by heat, as shown in the following experiments. In one series all the ingredients were mixed and the medium boiled for five minutes before pouring

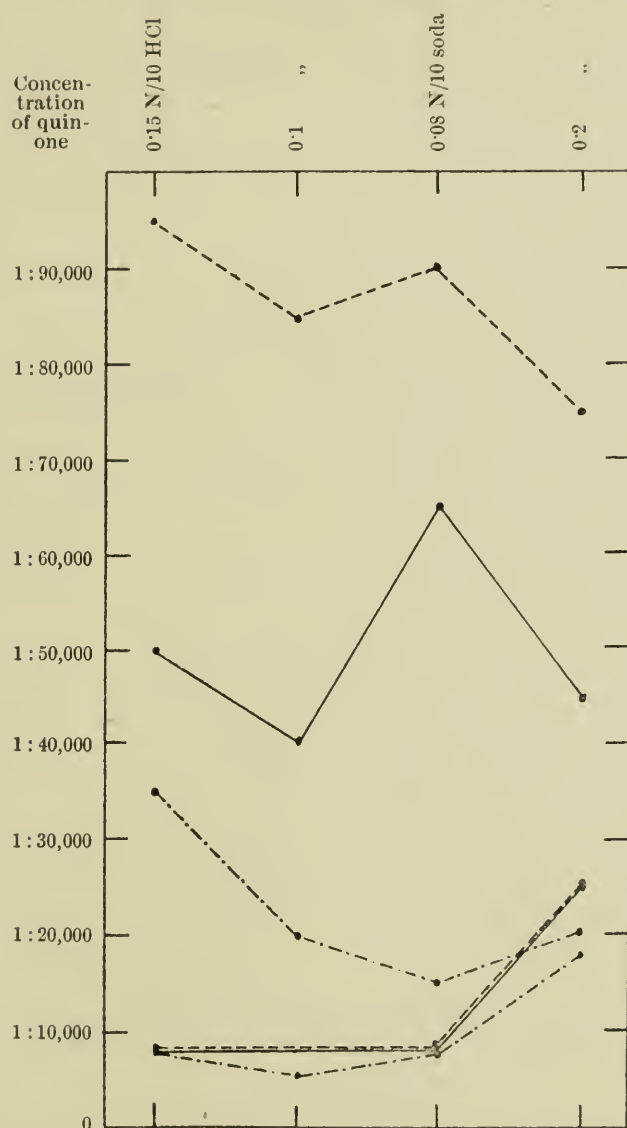


CHART VI. Showing the influence of boiling on the action of quinone. Upper lines indicate unheated series, lower lines boiled series.

----- *B. coli*                      ——— staphylococci  
 -.-.-.- *B. pyocyaneus*



*Action of Dyes on Bacteria*

the plates, while in the other the ingredients without the quinone were first mixed and boiled. After cooling to 45° C. the solution of quinone was added and plates immediately poured.

Composition of media						
	Meat extract	N/10 HCl	N/10 soda	Agar	Water	Solution of quinone
A.	1.0	0.15	—	2.0	1.35	0.5
B.	1.0	0.05	—	2.0	1.45	0.5
C.	1.0	—	0.08	2.0	1.42	0.5
D.	1.0	—	0.2	2.0	1.3	0.5

Concentration of quinone required to inhibit growth								
	Unheated quinone solution				Heated quinone solution			
	A	B	C	D	A	B	C	D
<i>B. coli</i>	1: 95,000	1: 85,000	1: 90,000	1: 75,000	1: 7,500	1: 7,500	1: 7,500	1: 25,000
Staph.	1: 50,000	1: 40,000	1: 65,000	1: 45,000	1: 7,500	1: 7,500	1: 7,500	1: 25,000
<i>B. pyocy-aneus</i>	1: 35,000	1: 15,000	1: 15,000	1: 15,000	1: 7,500	1: 3,000	1: 7,500	1: 15,000

*The action of quinone on cultures of various ages.*

Cultures made in meat extract 1.0 c.c., N/10 soda 0.8 c.c., and water 3.5 c.c., were incubated at 37° C. for one, three and ten days respectively. At the expiration of these periods 0.5 c.c. of a solution of quinone was added to each, and the tube returned to the incubator. 24 and 72 hours later one standard loopful was sown from each culture on agar, and incubated for 48 hours.

Cultures one day old							
Concentration of quinone	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
	<i>B. coli</i>	Staphylococci	Concentration of quinone	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 10,000	numerous	0	1: 1,000	0	numerous	0	0
1: 25,000	„	numerous	1: 2,500	0	„	numerous	0
1: 50,000	„	„	1: 5,000	numerous	„	„	numerous
1: 75,000	„	„	1: 7,500	„	„	„	„

Cultures three days old							
	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
	<i>B. coli</i>	Staphylococci	Concentration of quinone	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 10,000	numerous	0	1: 1,000	0	numerous	0	0
1: 25,000	„	0	1: 2,500	0	„	0	0
1: 50,000	„	0	1: 5,000	numerous	„	0	numerous
1: 75,000	„	0	1: 7,500	„	„	numerous	„
1: 100,000	„	0	1: 10,000	„	„	„	„

Cultures ten days old							
	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
	<i>B. coli</i>	Staphylococci	Concentration of quinone	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 10,000	0	0	1: 1,000	0	numerous	0	0
1: 25,000	few	0	1: 2,500	0	„	0	0
1: 50,000	numerous	0	1: 5,000	numerous	„	0	numerous
1: 75,000	„	0	1: 7,500	„	„	0	„
1: 100,000	„	0	1: 10,000	„	„	0	„

In the case of staphylococci it will be noticed that the older the culture the more affected it is by treatment with quinone. In the case of *B. coli* this phenomenon is not so evident. *B. pyocyaneus* is equally affected at all ages.

As in the case of homoflavine the results obtained by adding the solution of quinone to growing cultures are very different from those obtained when relatively small numbers of organisms are added to media containing a solution of quinone.

**The influence of numbers of organisms and of gelatin on the effects of homoflavine and of quinone.**

*The influence of numbers in the initial dose of organisms.*

Certain experiments illustrating the influence of variations in the numbers of the organisms in the initial dose on the effects of homoflavine and quinone were carried out simultaneously and are best considered in conjunction.

The culture medium (neutral to neutral red) consisted of meat extract 1.0 c.c., soda 0.8 c.c., water 3.42 c.c. and dye 0.5 c.c. In series A the concentration of homoflavine was 1 : 100,000 and in series B 1 : 250,000, in series C the concentration of quinone was 1 : 20,000, and in series D 1 : 50,000. Each series consisted of seven tubes to each of which one drop of an emulsion in distilled water of staphylococci from an agar culture grown at 37° C. for 24 hours was added. The first tube received a drop of a strong emulsion, the next of an emulsion of 1/10 strength, and the others of 1/100, 1/1000, 1/10,000, 1/100,000, 1/1,000,000 strengths. The organisms present in these tubes were estimated immediately by sowing standard loops (0.01 c.c.) of the cultures in melted agar and pouring plates, and in the same way the numbers were estimated, using dilutions when necessary, after 24, 48, 72 and 96 hours' cultivation at 37° C.

It will be seen that in the controls very active multiplication took place within 24 hours in all the cases tested.

In a concentration of 1 : 100,000 homoflavine, series A, a few of the organisms remained alive in tube 1, to which the greatest numbers were added, but none appeared to remain alive in the other tubes to which smaller numbers were added. At a concentration of 1 : 250,000 of homoflavine, series B, multiplication of the organisms occurred rapidly in tube 1, more slowly in tube 2, and some multiplication, after an initial diminution, in tubes 3 and 5. In the latter case the organisms were so few after 24 hours' incubation that no colonies appeared in the subculture made with 0.01 c.c. of the culture. In the other tubes no organisms appeared to survive. It seems probable therefore that, whereas in tubes 4, 6 and 7 all the organisms were destroyed in the stage when diminution in numbers is occurring, in tube 5 one or more survived, became accustomed to the conditions and subsequently multiplied.

A concentration of 1 : 20,000 of quinone, series C, inhibited growth in all cases, while with a concentration of 1 : 50,000, series D, after a great initial

*Action of Dyes on Bacteria*

diminution rapid growth occurred in tube 1, but no colonies ever appeared in subcultures from the other tubes.

No. of tube	Dilution of cocci	Immed. count per loop	Homoflavine Series A 1:100,000			Homoflavine Series B 1:250,000			Quinone Series D 1:50,000		
			24 48			24 48 72			24 48 72		
			hrs.			hrs.			hrs.		
1.	full	83,500	57	61	0	0	0	0	58	5,131,000	8,352,000
2.	1/10	7,904	0	0	0	0	0	0	0	0	0
3.	1/100	872	0	0	0	0	0	0	0	0	0
4.	1/1,000	94	0	0	0	0	0	0	0	0	0
5.	1/10,000	7	0	0	0	0	0	0	0	0	0
6.	1/100,000	1	0	0	0	0	0	0	0	0	0
7.	1/1,000,000	0.8	0	0	0	0	0	0	0	0	0
Controls			Immediate			24 hours					
			1/10			7,904					
			1/1,000			872					
			1/10,000			7					
			1/1,000,000			0.8					

Several other experiments of this type gave similar results, showing that the concentration of the compound necessary to inhibit growth is greatly



influenced by the initial number of organisms present. Small numbers of staphylococci seem to be able to overcome the influence of homoflavine more easily than the influence of quinone.

*The influence of gelatin.*

Several series of experiments on the influence of gelatin on the effects of homoflavine and of quinone were carried out. The first series quoted is intended to illustrate the influence of gelatin in the presence of strong concentrations of these compounds.

In this experiment small quantities of gelatin, agar or albumin were added in some of the tubes, while other tubes in the series acted as controls. All were made neutral to neutral red. The tubes were sterilised by boiling and the solution of the compound, sufficient to make a final concentration of 1 : 1,000, the albumin and a drop of an emulsion of staphylococci added after cooling.

No. of tube	Meat extract	Salt solution (3.75 %)	N/10 soda	N/10 HCl	Gelatin (20 %)	Agar (2 %)	Egg albumin	Water	Solution of compound
1.	1.0	1.0	0.08	—	—	—	—	2.42	0.5
2.	1.0	1.0	0.17	—	1.0	—	—	1.23	0.5
3.	—	1.0	0.09	—	1.0	—	—	2.41	0.5
4.	1.0	1.0	0.08	—	—	0.5	—	1.92	0.5
5.	—	1.0	—	—	—	0.5	—	3.0	0.5
6.	—	1.0	—	—	—	—	—	3.5	0.5
7.	1.0	1.0	0.08	—	—	—	0.5	1.92	0.5
8.	—	1.0	—	—	—	—	0.5	3.0	0.5
9.	—	1.0	—	—	—	—	0.5	3.5	—
10.	—	1.0	—	—	—	—	—	4.0	—
11.	—	1.0	—	0.1	—	—	—	3.9	—
12.	—	1.0	1.0	—	—	—	—	3.0	—

Immediately after the introduction of the organisms and after 1, 5½ and 24 hours' incubation at 37° C. standard loopfuls were sown in agar plates, the colonies on which were counted after 48 hours' incubation, with the following results.

Tube	Homoflavine			Quinone		
	1 hour	5.5 hours	24 hours	1 hour	5.5 hours	24 hours
1.	280	0	0	0	0	0
2.	3,264	168	0	0	0	0
3.	5,886	1,136	0	0	0	0
4.	488	1	0	0	0	0
5.	4,000	37	0	0	0	0
6.	2,656	360	0	0	0	0
7.	1,072	0	0	0	0	0
8.	1,696	0	0	0	0	0
	Control tubes					
	1 hour	5.5 hours	24 hours			
9.	7,200	6,016	4,448			
10.	5,984	4,624	0			
11.	26	23	2			
12.	7,616	1,632	0			

The immediate cultures yielded an average of 9950 organisms per loop.

It will be seen that a rapid diminution in the number of organisms took



place in the presence of 1 : 1,000 homoflavine in tube 1, containing meat extract only, and in tube 4, containing meat extract and agar. The numbers diminished much less speedily in tube 2 containing meat extract and gelatin, tube 3 containing gelatin only, tube 5, containing agar without meat extract, and tubes 7 and 8, containing albumin. The numbers also diminished slowly in tube 6 containing salt solution. On the other hand in a concentration of 1 : 1,000 neither gelatin, agar, albumin or salt solution seems to retard the effects of quinone.

In the control tubes 10, 11, 12, containing neutral, acid and alkaline salt solution, very few or no living organisms were found after 24 hours' incubation, but numerous colonies were present in subcultures made at that time from tube 9, containing albumin.

The influence of various quantities of gelatin, with and without meat extract, was studied in another series of experiments.

Tubes containing media of the following composition were prepared and sterilised by boiling. The solution of the compound and one drop of a staphylococcus emulsion were added when cool, and the cultures incubated at 37° C. Immediate counts showed that the cultures contained an average of 2,016 organisms per loop.

		Meat extract	Gelatin (20 %)	N/10 soda	Water	Solution of compound
Controls	1.	—	2.0	0.18	2.82	—
	2.	—	1.0	0.09	3.91	—
	3.	—	0.5	0.045	4.45	—
	4.	—	2.0	0.18	2.32	0.5
	5.	—	1.0	0.09	3.41	0.5
	6.	—	0.5	0.045	3.95	0.5
Controls	7.	1.0	2.0	0.26	1.74	—
	8.	1.0	1.0	0.17	2.83	—
	9.	1.0	0.5	0.125	3.375	—
	10.	1.0	2.0	0.26	1.24	0.5
	11.	1.0	1.0	0.17	2.33	0.5
	12.	1.0	0.5	0.125	2.875	0.5

Of numbers 4, 5, 6, 10, 11 and 12 four tubes were prepared, two (*a* and *b*) receiving solution of homoflavine of such strength as to make concentrations of 1 : 1,000 and 1 : 10,000 and two (*c* and *d*) solutions of quinone to make concentrations of 1 : 1,000 and 1 : 10,000. The organisms present in the cultures were counted by transferring standard loopfuls to melted agar and pouring plates immediately and after 24, 48, 72 and 96 hours' incubation at 37° C.

The results of these experiments are tabulated below.

No. of tube	Controls		No. of tube	1:1,000			Homoflavine				Quinone							
				24 hrs.	48 hrs.	72 hrs.	No. of tube	1:10,000			No. of tube	1:1,000			No. of tube	1:10,000		
	hours	hours						hours	hours	hours		hours	hrs.	hrs.		hrs.	hrs.	hrs.
1.	496,000	747,000	4 (a).	0	0	0	4 (b).	0	0	0	4 (c).	0	0	0	4 (d).	0	0	0
2.	424,000	596,000	5 (a).	0	0	0	5 (b).	0	0	0	5 (c).	0	0	0	5 (d).	0	0	0
3.	268,000	408,000	6 (a).	0	0	0	6 (b).	0	0	0	6 (c).	0	0	0	6 (d).	0	0	0
7.	6,416,000	10,608,000	10 (a).	0	0	0	10 (b).	60,000	1,576,000	5,680,000	10 (c).	0	0	0	10 (d).	0	0	0
8.	6,272,000	9,712,000	11 (a).	0	0	0	11 (b).	488	141	217	11 (c).	0	0	0	11 (d).	0	0	0
9.	5,582,000	7,232,000	12 (a).	0	0	0	12 (b).	3	1	0	12 (c).	0	0	0	12 (d).	0	0	0

It will be seen that in the control tubes, 1, 2 and 3, containing gelatin without meat extract the staphylococci multiplied to a moderate extent, and the multiplication was greatest in tube 1 containing the largest amount of gelatin. Homoflavine and quinone in concentrations of 1 : 1,000 and 1 : 10,000 completely inhibited growth in gelatin alone (4 *a, b, c, d*, 5 *a, b, c, d*, and 6 *a, b, c, d*). In all the control tubes containing gelatin and meat extract (7, 8 and 9) great multiplication occurred, the numbers found bearing a relation to the amount of gelatin present. While homoflavine in a concentration of 1 : 1,000 completely inhibited growth in these media [tubes 10 (*a*), 11 (*a*), 12 (*a*)] multiplication occurred at a concentration of 1 : 10,000 in the tube 10 (*b*) containing the most gelatin. In tube 11 (*b*) containing less gelatin the organisms diminished in numbers but some remained alive. Almost complete inhibition occurred in tube 12 (*b*) containing the least gelatin. Quinone in both concentrations inhibited growth in all cases.

These experiments like the last show that the action of quinone is much less affected than the action of homoflavine by the presence of gelatin.

*The influence of homoflavine and quinone on the liquefaction of gelatin by B. pyocyaneus.*

Media of the following composition were prepared and sterilised by boiling. After cooling the solution of the compound and one drop of an emulsion of *B. pyocyaneus* in distilled water was added.

	Meat extract	Gelatin (20%) in distilled water	N/10 soda	N/10 HCl	Water	Solution of compound
A.	1.0	2.0	0.56	—	0.94	0.5
B.	1.0	2.0	0.26	—	1.24	0.5
C.	1.0	2.0	0.1	—	1.4	0.5
D.	1.0	2.0	—	0.1	1.4	0.5

The cultures were incubated at 37° C. and cooled daily in a stream of water to see if liquefaction had taken place. Subcultures made on the second day showed numerous colonies in all the tubes, indicating that, under the conditions of these experiments, 1 : 1,000 concentrations of homoflavine and quinone did not completely inhibit the growth of *B. pyocyaneus*. The results are shown in the following table (p. 26).

It will be seen that the concentrations of homoflavine used, although they do not destroy the organisms, tend to inhibit the action or the production of the liquefying ferment most strongly when the medium is alkaline, while these concentrations of quinone act in the same manner most efficiently in series C, which is nearly neutral.

Other experiments of the same kind indicated clearly that the results were correlated with the number of organisms introduced. With a large initial dose liquefaction occurred in the 1 : 2,000 concentration of homoflavine on the fourth day in series A, and in the 1 : 1,000 concentration of quinone on the third day in series D.





Bashford, Hartley and Morrison (1917) in recording their experience of the action of acriflavine on wounds say that "The only favourable feature has been that the patient is apparently protected in some way from the absorption of toxic products." Possibly this protection is due to the influence of the dye on ferment action as illustrated in the experiment just quoted.

*Summary of experiments with homoflavine and quinone.*

	Homoflavine	Quinone
1 c.c. meat extract, 3.5 c.c. water, 0.5 c.c. solution of com- pound.	<i>B. coli</i> inhibited at concentration of 1 : 150,000, staphylococci of 1 : 300,000, <i>B. pyocyaneus</i> of 1 : 20,000.	<i>B. coli</i> inhibited at concentration of 1 : 90,000, staphylococci of 1 : 50,000, <i>B. pyocyaneus</i> of 1 : 7,500.
Alkaline meat ex- tract.	Efficiency greatly increased by small additions of N/10 soda.	Efficiency decreases as alkalinity increases. Most efficient at or near neutrality.
1 c.c. meat extract, 2 c.c. agar, 1.5 c.c. water, 0.5 c.c. solu- tion of compound.	<i>B. coli</i> inhibited at a concentra- tion of 1 : 4,500, staphylococci of 1 : 10,000, <i>B. pyocyaneus</i> of 1 : 500. Efficiency much less than in meat extract.	<i>B. coli</i> inhibited at a concentra- tion of 1 : 90,000, staphylo- cocci of 1 : 70,000, <i>B. pyocy- aneus</i> of 1 : 25,000. Efficiency nearly the same as in meat extract.
Alkaline meat ex- tract agar.	Efficiency increases with alka- linity.	Efficiency decreases as alkalinity increases.
Growing cultures.	Much greater concentrations re- quired to inhibit growing cul- tures than organisms added to fresh media. The oldest cultures are the most susceptible to prolonged ac- tion.	Action in this respect same as that of homoflavine.  Action in this respect same as that of homoflavine.
Heating to 100° C.	Heating has little effect on its action.	Heating very greatly decreases the efficiency.
Initial dose of organ- isms.	Efficiency decreases in relation to the numbers of organisms added. With moderate num- bers there is first a diminution and then a multiplication.	Relation of efficiency to numbers less marked than with homo- flavine, and less tendency to multiplication after initial de- crease.
Gelatin in meat ex- tract.	Gelatin in the presence of strong concentrations of the dye re- tards the inhibitory action. In moderate concentrations mul- tiplication occurs if sufficient gelatin present.	Action little affected by the presence of gelatin.
Liquefaction of gela- tin by <i>B. pyocyaneus</i> .	Inhibited when medium alka- line.	Inhibited when medium neutral.
Egg albumin.	In strong concentrations of the dye retards the inhibitory action.	Action little affected.

### Crystal Violet.

As shown in Table I crystal violet has relatively little action on *B. coli* or *B. pyocyaneus*, and consequently the few additional experiments carried out with this dye were made with staphylococci.

Experiments made with media of the following composition show that this dye like homoflavine acts best when the medium is slightly alkaline.

	Meat extract	Composition of medium				Solution of dye	Concentration neces- sary to inhibit the growth of staphylococci
		Agar	N/10 soda	N/10 HCl	Water		
A.	1.0	2.0	—	0.15	1.35	0.5	1 : 1,500,000
B.	1.0	2.0	0.08	—	1.42	0.5	1 : 3,500,000
C.	1.0	2.0	0.58	—	0.92	0.5	1 : 5,000,000

In neutral meat extract the growth of staphylococci was found to be inhibited by a concentration of 1 : 10,000,000, but the precise limits were not worked out.

Crystal violet acts more efficiently on staphylococci growing in meat extract than on ox serum.

### Conclusions.

In cultures the effects of homoflavine and quinone, the two compounds most thoroughly investigated, vary on each species of organism with every change in the composition of the medium, whether the change is brought about by altering the proportion of any constituent or by the introduction of fresh constituents, and also with variations in the numbers and age of the organism. Again in each medium the concentration of the compound which inhibits each species of organism differs, and it is probable that yet other concentrations are required when mixed cultures are employed, though no experiments have been made to establish this point.

In wounds the conditions are more complex than in cultures. The conditions prevailing in no two wounds are likely to be identical, and in every wound the conditions are constantly altering, not only in regard to the chemical constituents of the fluids but also in regard to the numbers of organisms and the species and the relationships between them.

The work of Douglas, Fleming and Colebrook (1917) indicates "that bacterial symbiosis may play a very important rôle in wound infections." "Streptococci multiply much more rapidly when grown in symbiosis with various bacteria, amongst these being the group of diphtheroid bacilli which are present in practically every infected wound from the earliest stage until cicatrization is complete."

The conditions prevailing in a wound would be more closely simulated if frequent small additions of food substance were made to cultures. Under these conditions much greater concentrations of homoflavine would undoubtedly be required to cause complete inhibition of growth.



If any arguments based on results<sup>1</sup> in cultures of the type employed may be applied to wounds or other lesions associated with bacteria the following conclusions seem to be permissible in view of the experiments which have been quoted.

(1) No satisfactory results may be expected from the use of a dye or allied compound as a bactericidal agent unless the wound has been thoroughly cleansed before its application, since the complex organic fluids present are likely to interfere with the action of the solution.

(2) The most beneficial results are likely to be obtained if the solution of the compound is made in a fluid of the reaction at which the compound acts most efficiently, provided such a reaction is not in itself harmful to the tissues.

(3) Some compounds are more efficient than others against certain species of bacteria. In each case the dyes or other compounds used should possess special efficiency against the organisms ascertained to be present in the wound or lesion.

(4) Solutions of these compounds are most likely to produce satisfactory results if used in the very early stages of infection when the organisms are few, and unaccustomed to the new conditions in which they find themselves. Apart from killing the organisms or checking their growth the inhibitory action of certain of these compounds on some of the ferments would tend to render the conditions less favourable for bacterial growth and to hinder the production of toxic substances. When the organisms are very numerous, growing rapidly accustomed to their surroundings and protected in the fluids, these compounds are likely to be much less effective.

The last two conclusions based on culture experiments receive some support from clinical experience with acriflavine. Kellock and Harrison (1917) say that "an interesting point noticed lately has been that the antiseptic flavine appears to have no effect on *B. pyocyaneus*," and Taylor (1917) makes the following statement: "A large number of bacteriological examinations of wounds under treatment with different solutions has been recorded and instances of the specific action of certain dressing solutions demonstrated."

Drummond and McNee (1917) conclude that "flavine has many advantages as a *primary* treatment," but state that it is "not a success in the later stages." Pearson (1918) states that "in cases where infection and sepsis are active and uncontrolled the use of flavine following suitable operative measures has no beneficial effect on the subsequent progress of the case in so far as the control of sepsis is concerned."

<sup>1</sup> "It is generally recognized that the testing of substances for their antiseptic or germicidal properties is fraught with innumerable pitfalls and that by varying the conditions of testing almost any kind of result may be obtained" (Dakin and Dunham, 1917).

## APPENDIX

## CLINICAL OBSERVATIONS.

Several observers very kindly tested the action on wounds of a solution of 1 : 10,000 homoflavine and 1 : 100,000 crystal violet in distilled water. Many of these tests were carried out with the greatest care, the descriptions being accompanied by charts showing the previous treatment, the age and extent of the lesion, the rate of healing, the organisms found in smears and in cultures, etc. In some instances the progress of two similar wounds in the same individual, one treated with the solution mentioned and one with some other antiseptic, was compared. The results are described as "dramatic," "very successful," "successful," "moderate success" and "unsuccessful."

Careful though many of these inquiries have been they have not revealed the causes of the very divergent results, which occurred in the experience of several observers, and consequently it does not seem necessary to publish the cases in detail.

All the observations were made on relatively old wounds, and though they afford no evidence regarding the efficiency of the solution in the primary treatment of wounds, they show that old, infected wounds may be treated sometimes with "very successful" results. To ascertain the conditions under which successful results may be expected in such lesions requires combined bacteriological and chemical investigations on a series of suitable cases. Bacteriologically the species, number, rate of multiplication and symbiotic relationships of the organisms, and chemically the reactions of the fluids, the substances which inhibit the actions of the compounds, and especially the products of tissue decomposition, due to bacterial or tissue ferments, available as food material for the various species of bacteria, influence the results.

Apart from wounds this solution was employed with varying results in the treatment of gonorrhoea. Satisfactory and even "dramatic" results were obtained in several cases of stomatitis, gingivitis and pyorrhoea.

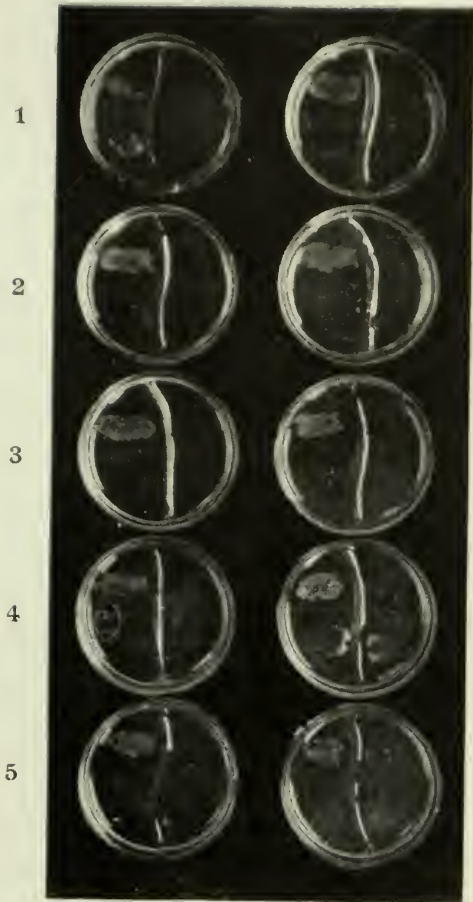
A 1 : 10,000 solution of quinone in distilled water has been tested on a small number of wounds with varying results. Like homoflavine it has given excellent results in some cases of pyorrhoea.

The three compounds differ so greatly in their powers of inhibiting the growth of such organisms as *B. coli*, staphylococci and *B. pyocyaneus* under varying cultural conditions that it might be desirable to test the action on wounds of a solution in distilled water of 1 : 10,000 homoflavine, 1 : 10,000 quinone and 1 : 100,000 crystal violet.

Pharmacologically these compounds are very inert, as shown by experiments which Professor A. R. Cushny, F.R.S., Dr W. E. Dixon, F.R.S., and Dr D. Cow very kindly carried out.



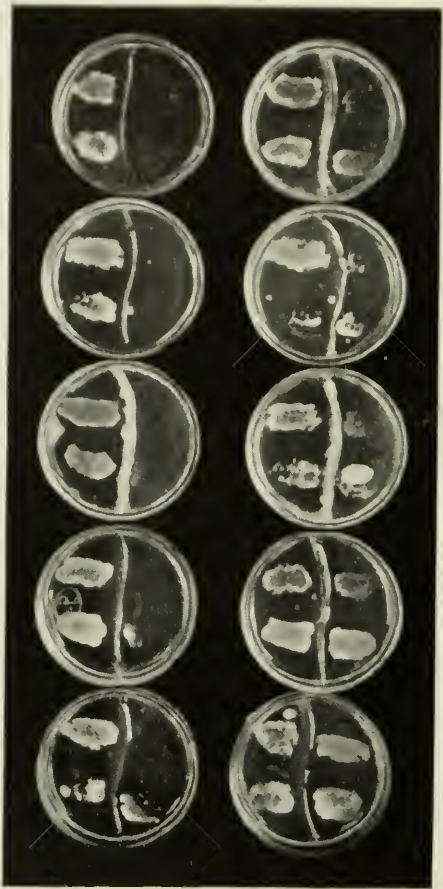
2-  
20



Figs. 1—10

6  
7  
8  
9  
10

11  
12  
13  
14  
15



Figs. 11—20



Fig. 21

## EXPLANATION OF PLATE.

Figs. 1-20. Photographs to show the differences of growth on agar after 24 hours' and after 48 hours' incubation at 37° C.

Figs. 1-10 represent Petri dishes divided according to Churchman's (1912) method after 24 hours' incubation at 37° C. The left side in each case is the control, and contains a medium composed of agar 2 c.c., ox pancreas extract<sup>1</sup> 0.5 c.c., distilled water 1.0 c.c., and N/10 soda 0.5 c.c. The right side contains a medium composed of agar 2 c.c., ox pancreas extract 0.5 c.c., distilled water 0.6 c.c., N/10 soda 0.5 c.c., and the solution of homoflavine 0.4 c.c. The concentration of homoflavine in dish 1 was 1 : 100,000, in dish 2 1 : 200,000 and so on up to 1 : 1,000,000 in dish 10.

An emulsion of *B. coli* in distilled water was streaked across the upper part of each side of each dish and an emulsion of staphylococcus across the lower part of each side.

Figs. 1-10 show the growth of *B. coli* in each case on the control side, but in most cases no visible growth of the staphylococcus. On the right side no visible growth of either organism has occurred in 24 hours at 37° C.

Figs. 11-20 represent the same dishes after 48 hours' incubation. On the left or control sides considerable growths of both *B. coli* and staphylococcus are seen. On the right sides a slight growth of staphylococcus is seen first in fig. 14 with a concentration of 1 : 400,000 homoflavine, and it becomes more marked in the later dishes, being almost equal to the controls for fig. 16 (homoflavine 1 : 600,000) onwards. Growth of *B. coli* is seen first in fig. 16 with a concentration of 1 : 600,000 homoflavine. In concentration of 1 : 900,000 and 1 : 1,000,000 (figs. 19, 20) the growths are equal to the controls.

These photographs indicate sufficiently the necessity for adopting a rigid time limit in recording the results of such experiments.

Fig. 21. A Petri dish on the bottom of which two vertical streaks of plain agar were made (seen as light coloured vertical bands), and allowed to set. Over them was poured a medium composed of meat extract 1 c.c., agar 2 c.c., N/10 soda 0.08 c.c., distilled water 1.42 c.c. and 0.5 c.c. of a solution of homoflavine, making a final concentration of 1 : 17,000.

An emulsion of *B. coli* was streaked transversely across the upper part of the dish, and the colonies of this organism are seen stretching across the dish. Across the lower part of the dish an emulsion of staphylococcus was streaked. It will be noticed that the colonies of this organism are growing only over the streaks of plain agar.

The photograph represents the condition after 48 hours' incubation at 37° C.

<sup>1</sup> Numerous experiments with pancreas and other organ extracts were made, but have not been quoted, as new factors are introduced which have not been worked out sufficiently.

## REFERENCES.

- BASHFORD, E. F., HARTLEY, J. N. J. and MORRISON, J. T. (29. xii. 1917). A study of fifty cases treated with flavine. *Brit. Med. Journ.* II. 849.
- BROWNING, C. H. and GILMOUR, W. (1914). Bactericidal action and chemical constitution with special reference to basic benzol derivatives. *Journ. of Path. and Bact.* XVIII. 144.
- BROWNING, C. H., GULBRANSEN, R., KENNAWAY, E. L. and THORNTON, L. H. D. (1917). Flavine and Brilliant Green. *Brit. Med. Journ.* I. 73.
- BROWNING, C. H., GULBRANSEN, R. and THORNTON, L. H. D. (1917). The antiseptic properties of Acriflavine and Proflavine, and Brilliant Green. *Brit. Med. Journ.* II. 70.
- CHURCHMAN, J. W. (1912). The selective bactericidal action of gentian violet. *Journ. of Exp. Med.* XVI. 221.
- (1913). The selective bactericidal action of methylene blue. *Journ. of Exp. Med.* XVIII. 579.
- (1913). The selective bactericidal action of stains closely allied to gentian violet. *Journ. of Exp. Med.* XVII. 374.



- CHURCHMAN, T. W. and HERZ, L. F. (1913). The toxicity of gentian violet and its fate in the animal body. *Journ. of Exp. Med.* xviii. 579.
- CHURCHMAN, J. W. and MICHAEL, W. H. (1912). The selective action of gentian violet on closely allied bacterial strains. *Journ. of Exp. Med.* xvi. 822.
- DAKIN, H. D. and DUNHAM, E. K. (1917). The relative germicidal efficiency of antiseptics of the chlorine group and acriflavine and other dyes. With observations on the rational testing of antiseptics. *Brit. Med. Journ.* ii. 641.
- DOUGLAS, S. R., FLEMING, A. and COLEBROOK, L. (1917). Studies in wound infections; On the question of bacterial symbiosis in wound infections. *Lancet*, i. 605.
- DRUMMOND, H. and McNEE, J. W. (1917). On the treatment of a series of recently inflicted war wounds with flavine. *Lancet*, ii. 640.
- FLEMING, A. (1917). The physiological and antiseptic action of flavine (with some observations on the testing of antiseptics). *Lancet*, ii. 341.
- HEWLETT, R. T. (1917). Note on the germicidal powers of flavine. *Lancet*, ii. 492.
- KELLOCK, T. H. and HARRISON, C. R. (1917). A note on the occurrence of "blue pus" in septic wounds. *Lancet*, ii. 605.
- KRUMWIEDE, C. and PRATT, T. S. (1914). Observations on the growth of bacteria in media containing various aniline dyes. *Journ. of Exp. Med.* xix. 20.
- MORGAN, W. P. (1918). Observations on the action of acriflavine and proflavine. *Lancet*, i. 256.
- NICHOLLS, L. (1917). The chemical affinities of the cholera vibrios. *Lancet*, ii. 563.
- PEARSON, W. (1918). Clinical observations on the effects of flavine in wound treatment. *Lancet*, i. 370.
- TAYLOR, K. (1917). Specificity in antiseptics. *Lancet*, i. 294.
- TEAGUE, O. (1918). The toxicity of Victoria Blue 4 R. for *B. paratyphosus* A, *B. paratyphosus* B and *B. enteritidis*. *Journ. Bact.* iii. 1.
- WRIGHT, J. H. (1917). The importance of uniform culture media in the bacteriological examination of disinfectants. *Journ. of Bact.* ii. 315.



# THE TESTING OF ANTISEPTICS IN RELATION TO THEIR USE IN WOUND TREATMENT<sup>1</sup>.

BY C. H. BROWNING AND R. GULBRANSEN.

(From the Bland-Sutton Institute of Pathology, the Middlesex Hospital.)

IN previous reports (Browning, Gulbransen, Kennaway and Thornton) attention was directed to the value of certain experimental tests of antiseptics as an indication of their practical value in the treatment of infected wounds, namely the estimation of antiseptic potency in the presence of serum and the relationship between this property and toxicity to tissues. In regard to the latter factor it was pointed out that toxicity must be investigated on a wide basis; thus determinations were made of the effect of antiseptics on leucocytes, as shown by alterations in their phagocytic properties, and on an epithelial membrane, *e.g.* the conjunctiva, as evidenced by the production of inflammation, and also the toxicity to the body as a whole was estimated in cases where the substance was readily absorbed (see Table I). It was emphasised,

Table I.

Substance	Maximum non-lethal dose for 20 gram mouse	Bactericidal concentration for			
		<i>Staph. aureus</i> in		<i>B. coli</i> (Escherich) in	
		0.7 % peptone water	Serum	0.7 % peptone water	Serum
Diamino-acridine sulphate (proflavine)*	0.003 gram	1 : 20,000	1 : 200,000	1 : 4,000	1 : 100,000
Diamino-methyl acridinium chloride (acriflavine)*	0.0006 „	1 : 20,000	1 : 200,000	1 : 1,300	1 : 100,000
Phenol	0.006 „	1 : 250	1 : 250	1 : 500	1 : 500
Mercury perchloride	0.0001 „	1 : 1,000,000	1 : 10,000	1 : 1,000,000	1 : 10,000

*Method of the Tests.* The toxicity for mice was determined by injecting watery solutions subcutaneously, the dose being so arranged that 20 gm. mouse received a volume of 1 c.c.: to animals of other weights corresponding volumes were given, but mice not exceeding the limits of 15–25 gm. were selected for the tests.

\* It has been suggested that the increased bactericidal action of the flavines in serum is merely an instance of a general enhancement of toxicity due to serum; as might be anticipated the injection of the dose in 80 per cent. serum instead of a watery solution does not, however, alter the fatal dose.

however, that the susceptibility of different tissues toward a given chemical compound may vary greatly and that this important character in substances destined for use as antiseptics requires much further investigation; the impossibility of using strychnine, even if it were a potent antiseptic, owing

<sup>1</sup> A Report to the Medical Research Committee.

to its specialised toxic action on the nervous system was cited. The result of our work on these lines pointed to the value of certain basic benzol derivatives—*brilliant green*—and acridine compounds—*acriflavine* and *proflavine*. Independent clinical observations (Ligat, James) supported the view that the sum total of tests which we had applied, constituted a trustworthy guide to the therapeutic value of these antiseptics and afforded indications as to the suitable means of application. In the interest of further progress it was obviously of great importance that the significance of suitable laboratory tests should be established as a guide to practical use, since such investigations constitute an essential preliminary to clinical trials. Subsequent observations by Drummond and McNee, Carslaw, Carslaw and Templeton, and Pilcher and Hull, have added greatly to our knowledge of the therapeutic value of the flavine compounds both for prevention and treatment of sepsis, and the clinical material on which the reports are based (over 5000 cases in Pilcher and Hull's series) indicates the representative nature of the tests. From these investigations the result emerges that, employed in conjunction with operative measures, salts of certain basic organic compounds, especially the flavines and also brilliant green, are capable of yielding valuable therapeutic results when applied by the relatively simple and rapid method of packing with gauze soaked in—not wrung out of—a solution of the antiseptic and an important feature of the flavines is that infrequent renewal in the wound suffices to maintain their effect. This may be advantageously provided for, on occasion, by a valved tube according to Kellock's method. The satisfactory action of infrequent renewal of the antiseptic seems clearly to be correlated with the continued activity of the flavine compounds in the presence of serum. The importance of interrupting the application of flavine after a certain stage, owing to an inhibition of healing—not to any destructive action on existing tissues—which may supervene, is a valuable practical contribution to our knowledge of the use of these compounds which has been established by the above-named clinical observers (see also Bashford, Hartley and Morrison). Several investigators, however, have published results of laboratory investigations tending to the conclusion that we have exaggerated the properties of the flavine compounds; accordingly, a number of points which have been raised relative to the action of these antiseptics on bacteria will be dealt with here. Their action on the tissues will be discussed elsewhere.

#### THE CHOICE OF A MEDIUM FOR TESTING ANTISEPTICS.

*Serum* was originally selected because serous exudate constitutes practically the diluent to which an antiseptic is exposed in a wound, both when recent and, also at a later stage when a granulating surface is mechanically cleansed from pus. The fact that serum intensifies the bactericidal action of the flavines, especially in the case of *B. coli*, has been met by the statement that serum is a poor culture medium and it has been suggested that the intensifying effect is merely a summation of the inhibitory properties of serum



and of the antiseptic. The error of these views becomes clearly apparent when one tests a number of specimens of serum and also a variety of antiseptics. Thus, while some specimens of ox serum afford a relatively poor medium for the proliferation of *B. coli*, others yield excellent growths. On what this depends has not been determined; the unsuitable sera are usually those which are practically colourless; but the difference has not been found to depend on factors such as length of contact with the clot or the addition of a trace of laked red corpuscles. The intensifying action of serum on the bactericidal property of flavine is well shown in the case of sera which are favourable to bacterial growth. The addition to the serum of an amount of trypsin (see Douglas and Colebrook) which augments the properties as a medium of unfavourable specimens, has not been found to alter distinctly its intensifying action for flavine<sup>1</sup>. The summation theory is further disproved by finding substances which are extremely potent antiseptics in watery medium, but whose action is greatly reduced by serum, *e.g.* in the case of brilliant green with *B. coli*—

Concentration in 0.7 % peptone water which kills = 1 : 130,000.

Concentration in serum which fails to kill = 1 : 5000 (result obtained by subculture after 48 hours: the control culture in serum without antiseptic yielded abundant growth).

If admixture with serum, in addition to merely diluting the antiseptic, also neutralises its effects, then frequent renewal of the substance will be essential in order to obtain efficient action in a wound. This is the case with all the common older antiseptics in watery solution except carbolic acid; such frequent renewal exposes the body as a whole to the danger of toxic effects should absorption occur; phenol and mercuric chloride are potent poisons when absorbed, hence their applicability is strictly limited. On the other hand, the hypochlorites have the great advantage of being converted into harmless compounds in contact with the tissues, but the "complicated hydraulic system," as Dakin, Lee, Sweet, Hendrix and Le Conte term it, which is necessitated by the unstable nature of these antiseptics in watery solution, in order to obtain efficient action, renders the suggestion of their general use practically a counsel of perfection. Brilliant green (first used by Leitch, see also Ligat and Webb) although diminished in action by serum, is highly potent and is scarcely absorbed; on the other hand, the flavines while absorbed are very little toxic, as was demonstrated by the fact that relatively large amounts could be administered intravenously (intravenous administration has now been practised on an extensive scale in cases of trench fever—Byam, Dimond, Sorapure, Wilson and Peacock).

As regards the action of other media, *pus* diminishes the antiseptic power

<sup>1</sup> Subsequent experiments have shown that the commercial preparation of trypsin which has been recommended for use varies considerably, thus two specimens acted as above stated; a third, however, in similar concentrations produced great alteration of the serum, as shown by abundant formation of tyrosine crystals without any bacterial contamination. Such profoundly altered serum ceased to give a marked intensification of the antiseptic effect of the flavines. This point is being investigated further.



of the flavines, as was shown in our first report (confirmed by Fleming, and Parry Morgan). *Defibrinated blood* diminishes the action as we demonstrated (confirmed by Fleming, Dakin and Dunham, Parry Morgan).

*Milk* diminishes the action (Hewlett), so does *minced meat* (Fleming), which also removes the bactericidal properties of blood serum. Dakin and Dunham have used further a mixture of watery muscle extract with serum. It would, of course, be of great value if an antiseptic were to act equally well in all media, but, this is probably impossible and even if an antiseptic could be disseminated in potent concentration by the blood stream it would still fail to penetrate effectively into any considerable mass of necrotic tissue, hence operative interference must be an essential factor in wound treatment. As regards the use of these other media, whose effect in neutralising flavine seems to have been regarded as an important observation by some of the investigators above mentioned, no cogent argument has been adduced to show that, as compared with serum, all or any of them constitute a more rational test medium for ascertaining the value of antiseptics in wounds. Milk and minced meat are clearly of only remote application, while, as we have repeatedly pointed out, pus is not the medium in which an antiseptic is required to act. Before application of an antiseptic pus and necrotic tissue should be removed mechanically; the layer of granulation tissue into contact with which the solution then comes is widely different from pus in its physical characters. We have throughout endeavoured to employ tests which possess "heuristic" value, and consider that the test in serum has been shown to belong to this category, whereas no proof has been afforded that the others do. It has been pointed out that the flavines are compatible with hypertonic saline up to 5 % NaCl; hence a means was provided for regulating to some extent the amount of serous fluid in the wound. This combination of flavine with hypertonic saline has been advantageously employed by Pilcher and Hull.

#### METHODS OF TESTING ANTISEPTIC POTENCY—BACTERIOSTATIC ACTION IN RELATION TO TOXICITY (EFFECT ON PHAGOCYTOSIS).

We pointed out originally that the flavines and brilliant green bring about the death of bacteria comparatively slowly; but apart from lethal action, very great dilutions of these compounds are effective in checking bacterial multiplication, that is, they exhibit a high degree of *bacteriostatic* action, to use Gildersleeve's term, and so act as potent antiseptics *sensu stricto*. On the other hand, substances such as mercuric chloride, phenol and chloramine-T all produce their maximum effect rapidly (within two hours) and no significant action occurs subsequently, *i.e.* concentrations of the latter substances which fail to prove lethal quickly, exert little or no effect on the multiplication of surviving bacteria. In addition, when it is remembered that serum neutralises these antiseptics—except phenol—the need for frequent renewal becomes clear; but the high toxicity of mercuric chloride and of phenol (which is a

very weak antiseptic) in relation to their antiseptic potency preclude unrestricted renewal.

It appeared that bacteriostatic action was a most valuable property for wound therapy and, because the serous exudate in the wound did not neutralise the flavines, mere slowness of lethal action as determined *in vitro* mattered little; the organisms under the influence of the antiseptic were, so to speak, controlled from the beginning. A striking confirmation of this is afforded by the observations of Drummond and McNee and also of Carslaw and Templeton, who found that in spite of the presence of bacteria in wounds treated with flavine there is a notable absence of both the local and the general phenomena of inflammation and of septic infections. Experimental evidence bearing on the co-operation of antiseptics with the tissues in overcoming infection will be published elsewhere.

Methods of testing antiseptics *in vitro*, whose sole aim is to detect whether or not every viable organism has been destroyed, may fail altogether to afford a true estimate of the potency of a substance for therapeutic purposes. Thus, Fleming and Hewlett inoculated fluid medium with the mixture of organisms and antiseptic and so obtained no information as to the numbers of organisms which had been killed short of total sterilisation. We have always made subcultures from the antiseptic mixtures on to solid media, so as to determine, by means of the number of colonies, whether the bacteria had increased or decreased. As the inoculation dose of organisms we originally chose a relatively minute number; the medium is not thereby rendered turbid at the time of inoculation, accordingly, if the mixture develops opacity later on, this indicates that free proliferation of the organisms has occurred. It was felt, however, that the use of a minute inoculation exposed our work to the objection that the antiseptic could not inhibit larger numbers of bacteria; accordingly, our second report contained experiments with much larger amounts of organisms, and it was shown in the case of acriflavine and *B. coli* that a twenty-thousand-fold increase in the inoculation dose necessitated only about a two-and-a-half-fold increase in the amount of antiseptic required to produce complete sterilisation. However, without direct reference to this latter work, our results with the basic organic antiseptics have been ascribed by Fleming, Hewlett, and Dakin and Dunham to the use of minute doses of organisms, which have been stated to lead to an exaggeration of the potency of the flavines as compared with other compounds. Accordingly, further tests have been performed, using larger amounts of organisms in conformity with the experiments of Dakin and Dunham.

The following is an example:

A 24-hour agar slope culture of *Staphylococcus aureus* was suspended in 5 c.c. of 0.85 per cent. NaCl solution: 0.025 c.c. each of a 1:100,000 and 1:1,000,000 dilution of this suspension when plated on agar yielded respectively 175 and 21 colonies, hence it may be concluded that the stock suspension contained about 750 million organisms per cubic centimetre.



To a series of tubes each containing 1 c.c. ox serum (heated previously for  $\frac{1}{2}$  hour at 55° C.) 0.1 c.c. of the *Staphylococcus* suspension was added and also 0.1 c.c. of varying dilutions of the antiseptic in water ("strong inoculation" series). In the "weak inoculation" series the inoculation dose of staphylococci was 0.1 c.c. of a 1 : 1000 dilution of a 24-hour peptone water culture. After incubation of the mixtures of organisms and antiseptic at 37° C. for 24 hours a loopful from each tube was stroked on agar; which was then incubated for 48 hours. The results were as follows:

Table II.

*Results of Subculture from Mixtures of Staphylococcus aureus and Flavines after 24 hours' contact at 37° C.*

Concentration of antiseptic	ACRIFLAVINE		PROFLAVINE	
	Weak inoculation	Strong inoculation	Weak inoculation	Strong inoculation
1 : 400,000	Marked diminution	—	8 colonies	—
1 : 200,000	Sterile	—	Sterile	—
1 : 130,000	„	Diminution	„	Diminution
1 : 100,000	„	„	„	„
1 : 40,000	„	Sterile	„	About a dozen colonies
1 : 20,000	„	„	„	„ „ „
1 : 13,000	„	„	„	„ „ „
				(Sterile in 48 hours)
1 : 4,000	„	„	„	Sterile

Controls after incubation:

*Weak* inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 22 colonies.

*Strong* inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 36 colonies.

Parallel experiments with the *strong* inoculation in a medium containing 33 per cent. serum yielded the same result as those with full serum.

In this and the following table "Diminution" = diminished number of colonies in subculture as compared with control without antiseptic.

In all cases the culture tubes were well shaken before making subcultures, so that any fallacy due to spontaneous sedimentation is excluded.

It might be objected that a loopful was an insufficient sample to take from each specimen; but the fact that a loopful of a 1 : 100,000 dilution of the inoculated serum controls without antiseptic, after incubation, yielded several dozen (22–36) colonies (see also Table III), proves that there is here no fallacy. Since the large inoculation dose, as employed by Dakin and Dunham, imparts a marked turbidity to the mixture, it was considered important to determine the relative number of viable organisms present in the control tubes containing serum, but no antiseptic, not only at the commencement of the experiment, but also after incubation. In order to do this a series of decimal dilutions was prepared in both cases and a loopful from each was stroked on agar; this simple procedure may be recommended as of sufficient accuracy for practical purposes, while it saves time and materials consumed by plating a series of dilutions. The results after incubation of the plates for 48 hours were as follows:



Table III.

Dilution	Serum <i>plus</i> Weak inoculation subcultured		Serum <i>plus</i> Strong inoculation subcultured	
	at once	after 24 hours at 37° C.	at once	after 24 hours at 37° C.
Undiluted	20 colonies	Homogeneous stroke	Homogeneous stroke	Homogeneous stroke
1 : 10	—	Slight decrease in density of growth	Homogeneous stroke	Discrete but closely adjacent colonies
1 : 100	—	Discrete but closely adjacent colonies	Slight decrease in density of growth	„
1 : 1,000	—	Marked decrease in number of colonies	Discrete but closely adjacent colonies	„
1 : 10,000	—	55 colonies	„	Marked decrease in number of colonies
1 : 100,000	—	22 „	„	36 colonies

The results illustrated in Tables II and III show:

(1) Controls: the large amount of organisms, as used for inoculation by Dakin and Dunham, did not maintain itself, *i.e.* in spite of some proliferation, as evidenced by increase in turbidity of the culture, the large inoculation led to increased death of bacteria, so that viable organisms were more numerous at the beginning than at the end of the experiment. It is obvious, therefore, that this is not the most suitable amount of organisms for testing the properties of a progressively acting antiseptic, since under the conditions arranged by Dakin and Dunham the organisms diminish in the absence of any antiseptic.

(2) With *Acridine*, in spite of the enormous number of organisms in the strong inoculation series, the lethal concentration (1 : 40,000) was not more than five times that found with the weak inoculation; further, a concentration of 1 : 130,000 produced a definite lethal effect on the organisms in the strong inoculation series. In the case of *Proflavine* it is clear that the result for practical purposes is the same, but there is a longer range of persistence of viable organisms before complete sterility is attained. Thus a loopful from the *undiluted* mixture containing proflavine 1 : 40,000 yielded only a dozen colonies, whereas the control without antiseptic when diluted 1 : 100,000 yielded 36 colonies in a loopful; therefore there can be no question as to the powerful bactericidal action exerted by this high dilution of the antiseptic. Such an effect will fail to be observed when testing results merely by subculturing into fluid medium, as practised by Fleming and Hewlett, where one viable organism will yield a growth; this would account for their conclusion that our original findings were exaggerated.

The following is a similar experiment in which acridine and proflavine were tested with large and small amounts of *B. coli*.

Method as above: the strong inoculation contained about 5000 million more organisms than the weak inoculation. The results are shown in Table IV

The Control *weak* inoculation subcultured at once yielded 220 colonies in a loopful and after incubation 1 : 10,000, 1 : 100,000, and 1 : 1,000,000 dilutions yielded 65, 30 and 2 colonies in a loopful respectively.

Table IV.

*Results of Subculture from Mixtures of B. coli and Flavines after  
24 hours' contact at 37° C.*

Dilution	ACRIFLAVINE		PROFLAVINE	
	Weak inoculation	Strong inoculation	Weak inoculation	Strong inoculation
1 : 400,000	Growth	—	Diminution	—
1 : 200,000	A few colonies (sterile in 48 hours)	—	A few colonies (sterile in 48 hours)	—
1 : 100,000	do.	Growth	Sterile	A few colonies (sterile in 48 hours)
1 : 40,000	—	A few colonies (sterile in 48 hours)	„	Sterile
1 : 20,000	—	„	„	„
1 : 13,000	—	Sterile	„	„

Controls after incubation:

*Weak* inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 30 colonies.

*Strong* inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 1 colony.

Parallel experiments with 33 per cent. serum medium gave the same result as full serum in the case of the strong inoculation.

The Control receiving the *strong* inoculation yielded 118 colonies in a loopful of a ten-thousand-million-fold dilution at once, but after incubation for 24 hours a loopful of 1 : 10,000, 1 : 100,000 and 1 : 1,000,000 dilutions yielded 30, 1 and 0 colonies respectively; hence with *B. coli* the strong inoculation shows even a more marked disadvantage as a test method than in the case of staphylococcus, owing to spontaneous death of the organisms.

It is striking that in the case of *B. coli* as compared with *Staphylococcus aureus* the behaviour of acriflavine and proflavine seems to be reversed and the former now tends to cause the wider zone of bacteriostatic action before complete sterilisation is attained; whether this is the invariable result with the two substances we have not sufficient evidence to show, but the general result is the same with both compounds, viz. that the strong inoculation requires for practically complete sterilisation at the most  $2\frac{1}{2}$  times the minimum lethal concentration required by the weak inoculation.

Accordingly, great increase—many million-fold—in the number of organisms in the inoculation dose, causes merely an insignificant increase— $2\frac{1}{2}$ –5-fold—in the concentration of the flavines necessary for practically complete sterilisation with *Staphylococcus* and *B. coli*; further, the occurrence of bacteriostatic action in much higher dilutions is a characteristic property of these substances.

We would again specially emphasise the therapeutic value of substances, like the flavines, which possess such powerful bacteriostatic properties and which are at the same time relatively non-toxic to the tissues and are not neutralised by serum. We had previously drawn attention to the ratio

concentration of substance which inhibits phagocytosis  
concentration causing death of organisms in serum

as one means of measuring the suitability of antiseptics for acting in a wound. The significance which we attached to this *therapeutic coefficient* has been



questioned because the time of exposure of organisms and of leucocytes to the antiseptic was not the same (Fleming), but this criticism fails altogether to take account of the fact that, short of producing actual death of the organisms, bacteriostatic action is exerted by these antiseptics. Thus, although the bacteria may still be capable of proliferating when removed into culture medium and out of contact with the antiseptic, this is of little practical importance as compared with the fact that their activities are inhibited when the antiseptic is present. Wright has also recently emphasised as an "all important principle" that the actual state of bacteria under conditions unfavourable to their activity (as where they are in contact with leucocytes in his observations or with antiseptics in our work) is not accurately shown by a method which proceeds to place the organisms under the most favourable circumstances for producing a culture.

In the case of the flavine antiseptics there exists such a wide range between the weakest concentration which will suffice to inhibit and eventually to kill organisms in a serous medium on the one hand, and on the other, the great strength which is required to paralyse phagocytosis<sup>1</sup> (see our previous reports), that antiseptic action and the natural defensive processes can be expected to go on side by side. No other antiseptic which has been tested, affords results which justify the belief that with it such will occur to anything approaching the same extent.

Of late there has been a tendency to question the importance of the leucocytes as a defensive mechanism in infections. Thus, prominence has been given to the fact that organisms which have been ingested by leucocytes, may be protected from the lethal action of antiseptic solutions (Jones and Rous); further, dissemination of infection has been attributed to the transport of organisms enclosed in leucocytes, and recrudescence has been explained by renewed activity of phagocytosed bacteria. The recent important experiments of Alexander, however, serve to restore perspective to the view of the anti-infective properties of the leucocytes—this worker found that virulent pneumococci when incubated along with antipneumococcus serum and leucocytes for a few hours (six), became attenuated, although they were not killed, whereas neither the antiserum nor the leucocytes by themselves produced this effect on the organisms. Thus, there is a good reason for taking into consideration the action of any proposed therapeutic agent on the activity of leucocytes when considering the properties which will determine its efficacy in the treatment of infections.

<sup>1</sup> Parry Morgan has also investigated the occurrence of phagocytosis *in vitro* in the presence of acriflavine and, since marked agglutination occurred in the mixture, has expressed the opinion that possibly "the phagocytosis was more apparent than real and that the organisms merely adhered to the phagocytes and were not ingested." We did not consider it necessary to refer to this point in our first report, since we had found that similar agglutination occurred with other substances, but that owing to the toxic effect of the latter phagocytosis did not take place and there were then no appearances which resembled the ingestion of organisms by the leucocytes observed in the experiments with flavine; thus it was evident that phagocytosis occurred in the presence of flavine.



## THE TESTING OF ANTISEPTICS OF THE CHLORINE GROUP.

Dakin and Dunham have recently observed that "in making tests of the germicidal efficiency of any antiseptic there seems to be no good reason for not following the order of mixing the materials indicated by the conditions of practical use—namely, to add the antiseptic last to the inoculated medium." With reference to our work they proceed further, "the lethal concentration of chloramine-T in serum is stated to be 1 : 250 without reference to much lower concentrations already published by others. The discrepancy is due, in the main, to the fact that the antiseptic was added first to the medium and the organisms last." We have now repeated our experiments (a) in the manner postulated by Dakin and Dunham, and (b) in the way in which they were previously performed, *i.e.* adding the antiseptic to the serum and then adding the organisms within 2 to 3 minutes afterwards, *i.e.* following Dakin's own previous publication in which this statement appears, "*Determination of Germicidal Action*... A series of tubes each containing 5 c.c. of a solution of the substance at a progressively decreasing concentration is first of all prepared, and to each tube the organism is added... The tests carried out in the presence of blood serum were performed in the same way, only the liquid in the first series of tubes contained 50 per cent. of horse serum previously heated at 55°–56° C." The results in both series are identical and confirm our previous figure (see Table V). We have been able to show that the difference between our results and Dakin's is due mainly to the fact that Dakin originally employed 50 per cent. serum—later Dakin and Dunham used 33 per cent. serum—whereas in our experiments the antiseptic mixtures contained over 80 per cent. of serum (in our experience 80 per cent. heated ox serum is a more favourable medium for the growth of staphylococci than is 40–50 per cent. serum).

The following is an example:

Medium = ox serum—previously heated at 55° C. for  $\frac{1}{2}$  hour—in each tube 1 c.c., (a) of undiluted serum, (b) of serum diluted with an equal volume of 0.85 per cent. NaCl solution. Chloramine-T stock solution = 1 : 23.3 (the strength of this solution was verified by titration both immediately before and after the time of employment in the experiment). Inoculation dose of organisms = 0.1 c.c. of 1 : 1000 dilution of a 24-hour peptone water culture of *Staphylococcus aureus*. In series A the organisms were added to the medium first and then the chloramine solution; in series B the chloramine was added first and then the cocci after an interval of 2–3 minutes. As a control aeriflavine in undiluted ox serum was tested. Sub-cultures were made on agar after 24 hours' incubation at 37° C. The results are shown in Table V.

These results confirm our original figures, although in the present instance 20 times as many organisms were employed for the inoculation as previously. It may be noted here that to dismiss the use of small numbers of bacteria as unsuitable for testing the action of an antiseptic *in vitro*, since a similar number in a wound borders on "surgical sterility," is entirely beside the point.

Table V.

*Action of Chloramine-T on Staphylococcus aureus.*

Concentration of chloramine	A		B	
	50 per cent. serum	Full serum	50 per cent. serum	Full serum
1 : 700	+	+	+	+
1 : 466	-	+	-	+
1 : 325	-	+	-	+
1 : 233	-	-	-	-

Control: concentration of acriflavine in undiluted serum, 1 : 400,000 +,

1 : 200,000 -.

+ = growth in subculture on agar.

- = no growth in subculture.

In a wound one is dealing with mechanisms inimical to the organisms (due to the leucocytes, etc.), which do not operate *in vitro*, hence the small number of organisms introduced into the culture medium is by no means subjected to the unfavourable conditions which may prevail in a wound.

## VARIATIONS IN RESISTANCE OF ORGANISMS TO FLAVINES.

Acriflavine and proflavine are the most powerful antiseptics so far investigated for staphylococci and the ordinary types of *B. coli* in a serum medium. Certain strains of streptococci appear to be even more susceptible (Parry Morgan), which should be advantageous in view of the rôle of streptococci in infected wounds, although we did not find the "enterococcus" to possess more than average susceptibility. The existence of marked differences in the resistance of various species of organisms toward a particular antiseptic is now well known (see Browning). Drummond and McNec isolated from certain wounds organisms of coliform type which showed a high degree of resistance toward the flavines; we have had the opportunity of investigating three such strains and find that they all belong to a most unusual type of *B. coli*, which fails to form indol, also they are late lactose-fermenters; their culture reactions were as follows:

Motility +	<div> glucose lactose saccharose mannitol maltose </div>	<div> } acid and gas </div>	<div> dulcitol inositol </div>	<div> } no change </div>	<div> milk acid and late clot; indol negative (12 days): gela- tine not liquefied </div>

We have tested the action in serum of both acriflavine and proflavine on eleven types of *B. coli* and also on *B. pyocyaneus*, and *Urobacillus septicus*;



the resistant strains being also included in the series; the results were as follows:

<i>B. coli</i> (Escherich)	}	No visible growth in serum <i>plus</i> acriflavine or proflavine 1 : 200,000 after 48 hours at 37° C.
<i>B. No. 71</i>		
<i>B. Schafferi</i>		
<i>B. Grünthal</i>		
<i>B. neapolitanus</i>		
<i>B. vesiculosus</i> (2 strains)		
<i>B. lactis aerogenes</i> (2 strains)		
<i>B. Morgan I</i>		
<i>B. paracoli</i> type		
<i>B. coscoroba</i>		
<i>B. No. 67</i> (inositol fermenter)	}	
<i>Urobacillus septicus</i> ( <i>proteus</i> class)		

Resistant (non-indol forming) coliform *B.* (3 strains)—visible growth in serum *plus* antiseptic 1 : 40,000; none in 1 : 20,000.

*B. pyocyaneus* (2 strains): visible growth in serum *plus* antiseptic 1 : 20,000; none in 1 : 10,000.

Inoculation dose in each case 0.1 c.c. of 1 : 1000 dilution of a 24-hour peptone water culture to 1 c.c. medium.

Controls without antiseptic all yielded abundant growth, with marked turbidity of the medium.

Parry Morgan, and Bashford, Hartley and Morrison also mention resistant coliform bacilli, but give no details as to their characters. It would, therefore, be quite misleading to suggest that the typical colon bacilli of faecal origin present such resistance to flavine antiseptics as to invalidate their use generally in wounds containing coliform organisms. *B. pyocyaneus* evidently belongs to the types most resistant to flavine, which has also been observed clinically (Kellock and Harrison), but this organism appears to be relatively unimportant as a pathogenic agent in wounds. It is interesting that *B. pyocyaneus* has also been found resistant to hypochlorites (Taylor).

#### SUMMARY AND CONCLUSIONS.

(1) *The antiseptic and bactericidal properties of Flavines and Brilliant Green.* Extended investigations have confirmed the original values. It has been shown that the inferior potencies recorded by certain other workers depend on the use of methods unsuited for the observation of antiseptic properties, *i.e.* they fail to detect inhibition of bacterial activity *i.e.* bacteriostatic action, which is exhibited to a marked degree by flavine and brilliant green.

(2) For the therapy of a local bacterial infection, as in a wound, such bacteriostatic action is of great value. It is not essential that the chemical



agent should by itself actually kill the organisms. Highly successful results can be obtained by a co-operation of the antiseptic and the tissues, so that the pathogenic action of the organisms is restrained. The flavines in virtue of their low toxicity to mammalian tissues and their high bacteriostatic power are therefore specially suited to act as local therapeutic agents. In addition, the fact that they are not neutralised by admixture with serum enables them to be applied clinically by a relatively simple method which does not necessitate frequent renewal.

(3) The "fundamental error" to which the method of testing chlorine antiseptics originally practised by Dakin, is liable, and which Dakin and Dunham have drawn attention to, has been shown not to affect our previous results with Chloramine-T. The difference between our values and the others is due to the fact that we employed 80 per cent. serum in the test medium, which is much more active in neutralising this antiseptic than is 33 to 50 per cent. serum employed by Dakin and his co-workers.

## REFERENCES.

- ALEXANDER. *Journ. of Med. Res.* vol. XXXVII. 1918; p. 471.  
BASHFORD, HARTLEY and MORRISON. *Brit. Med. Journ.* Dec. 29, 1917.  
BROWNING. *Applied Bacteriology*, London, 1918, pp. 88, 92.  
BROWNING, GULBRANSEN, KENNAWAY and THORNTON. *Brit. Med. Journ.* January 20, 1917 (I Report).  
BROWNING, GULBRANSEN and THORNTON. *Brit. Med. Journ.* July 21, 1917 (II Report).  
BYAM, DIMOND, SORAPURE, WILSON and PEACOCK. *Journ. Roy. Army Med. Corps*, Nov. 1917.  
CARSLAW. *Journ. Roy. Army Med. Corps*, October, 1917.  
CARSLAW and TEMPLETON. *Lancet*, May 4, 1917.  
DAKIN. See *Proc. Roy. Soc. B.* LXXXIX. 1916, p. 232.  
DAKIN and DUNHAM. *Brit. Med. Journ.* Nov. 17, 1917.  
DAKIN, LEE, SWEET, HENDRIX and LE CONTE. *Journ. Amer. Med. Assoc.* LXIX. p. 27, 1917.  
DOUGLAS and COLEBROOK. *Lancet*, July 29, 1916.  
DRUMMOND and MCNEE. *Lancet*, October 27, 1917.  
FLEMING. *Lancet*, September 1, 1917.  
HEWLETT. *Lancet*, September 29, 1917.  
JAMES. *Journ. Roy. Army Med. Corps*, March, 1917.  
JONES and ROUS. *Journ. Exp. Med.* XXIII. p. 601, 1916.  
KELLOCK. *Lancet*, 1917, II. p. 348.  
KELLOCK and HARRISON. *Lancet*, October 20, 1917.  
LEITCH. *Brit. Med. Journ.* February 12, 1916.  
LIGAT. *Brit. Med. Journ.* January 20, 1917.  
PARRY MORGAN. *Lancet*, February 16, 1918.  
PILCHER and HULL. *Brit. Med. Journ.* February 9, 1918; also Hull, *Surgery in War*, London, 1918, p. 67.  
TAYLOR. *Arch. d. Méd. et Pharm. Milit.* Paris, vol. LXVII. 1918, p. 48.  
WEBB. *Brit. Med. Journ.* June 30, 1917.  
WRIGHT. *Lancet*, January 26, 1918.

# RECENT ADVANCES IN THE DIFFERENTIATION OF LACTOSE-FERMENTING (GAS-PRODUCING) BACILLI, WITH SPECIAL REFERENCE TO THE EXAMINATION OF WATER AND FOOD PRODUCTS.

BY DENYS R. WOOD, F.I.C.,

*Public Analyst and Bacteriologist, Somerset County Council.*

*(From the Somerset County Public Health Laboratory.)*

IN recent years a considerable amount of work has been done by American bacteriologists on the differentiation of lactose-fermenting (gas-producing) organisms.

Rogers, Clark and Davis (1914) showed that by refined technique these organisms could be sharply differentiated into two main types by the estimation of the ratio of carbon dioxide to hydrogen produced in glucose media: those producing gases in the ratio  $\text{CO}_2 : \text{H}_2 =$  about 1.06 and characterised by the large proportion of strains producing indol, and those producing gases in the ratio  $\text{CO}_2 : \text{H}_2 =$  over 1.5 and characterised by the low proportion of strains producing indol.

Subsequently Rogers, Clark and Evans (1914) showed that practically only those organisms producing gases in the low ratio ( $\text{CO}_2 : \text{H}_2$ ) were to be found in bovine faeces, while in milk about 50 % belong to the low and 50 % to the high ratio ( $\text{CO}_2 : \text{H}_2$ ) type, and drew the inference that source of the latter must be sought elsewhere than in bovine faeces.

Later Rogers, Clark and Evans (1915) examined 166 cultures of glucose fermenters derived from various kinds of grain, 160 of these fermented lactose also, and 95 % of the lactose-fermenters were found to belong to the high ratio ( $\text{CO}_2 : \text{H}_2$ ) type. The further inference was therefore drawn that the high ratio type in milk might be derived from grain.

Clark and Lubs (1915) showed that the gas ratio ( $\text{CO}_2 : \text{H}_2$ ) varies inversely with the limiting hydrogen ion concentration attained in standard glucose peptone media containing a standard amount of buffer substance<sup>1</sup>, the low ratio type producing a hydrogen ion concentration equal to

$$1 \times 10^{-5} \bar{N} - 2 \times 10^{-5} \bar{N},$$

and the high ratio type a hydrogen ion concentration equal to

$$7.8 \times 10^{-7} \bar{N} - 0.1 \times 10^{-7} \bar{N},$$

<sup>1</sup> Composition of Clark and Lubs medium: glucose 0.5 %, peptone (Witte) 0.5 %, dipotassium phosphate  $\text{K}_2\text{HPO}_4$  0.5 %.



and showed moreover that these concentrations can be distinguished by means of the indicators methyl-red and *p*-nitro-phenol, and thereby rendered a simple method available for the differentiation of these two main types suitable for routine bacteriological examinations.

Levine (1916) correlated the Voges-Proskauer reaction with the limiting hydrogen ion concentration, having found that organisms producing a low concentration gave the Voges-Proskauer reaction while those producing a high concentration did not, and later, in conjunction with Weldin and Johnson (1917), devised an improvement in the original reaction accelerating the oxidation process with hydrogen peroxide. Myrtle Greenfield (1916) studied 432 cultures of lactose-fermenters from surface water, ground water, natural and artificial ice, and found that 138 (or 32 %) produced a low concentration of hydrogen ions. All these gave the Voges-Proskauer reaction, while none of those cultures producing a high concentration of hydrogen ions gave a positive V.-P. reaction.

Other workers in America have obtained similar results confirming the correlation of the Voges-Proskauer reaction with the limiting hydrogen ion concentration.

For convenience organisms producing high and low concentrations of hydrogen ions will be referred to in this paper as methyl-red positive ("M.R. +") and methyl-red negative ("M.R. -") respectively, and organisms giving and not giving the Voges-Proskauer reaction as "V.-P. +" and "V.-P. -."

As regards the distribution of the "M.R. +" and "M.R. -" types the following additional observations have been recorded:

Levine (1916) examined lactose-fermenters from the faeces of the horse (19), pig (21), cow (20), sheep (22), and man (25) and found none of the "M.R. -" type out of this total of 107.

Rogers, Clark and Lubs (1916) tested 113 cultures from human faeces and found only six (or 5.6 %) of the "M.R. -" type<sup>1</sup>. Rogers (1916) examined 137 cultures from surface water, 66 % of which were found to belong to the "M.R. -" type. The "M.R. +" types were occasionally found in springs in which there was no evident source of contamination, but were especially abundant in polluted waters. Johnson (1916) examined 363 coli-like organisms derived from soil and found 72 % of the "M.R. -" type.

Burton and Rettger (1917) found the predominant gas formers in soil were of the "V.-P. +" type and liquefied gelatine, *i.e.* were of the *B. cloacae* type. Out of 193 non-sporing lactose-fermenters 76 % were "V.-P. +" and liquefied gelatine, 5 % were "V.-P. +" and did not liquefy gelatine, and 19 % were "V.-P. -". He used the M.R. reaction also but regarded the V.-P. reaction as the more reliable with peptones other than Witte's.

<sup>1</sup> In a recent paper Rogers, Clark and Lubs (1918) record the isolation of 46 "M.R. -" strains from a collection of 177 derived from human faeces. The majority of these however were obtained by special methods, and 31 were obtained from a single specimen.



Levine (1918) gives the relative proportion of strains in soil as *cloacae* 49.7 %, *aerogenes* 30.5 %, and "M.R. +" types 18.7 %.

Observations of the Voges-Proskauer reaction have been recorded by a few investigators in this country, and, although it has not been regarded as altogether reliable, it is interesting to compare the results obtained with those briefly summarised above.

MacConkey (1906) recorded the examination of 107 cultures of non-chromogenic lactose-fermenters isolated from milk 31 of which (or 29 %) gave the Voges-Proskauer reaction. The same author (1909) examined 497 cultures from various sources including human and animal faeces, sewage, pond-water, roof washings, soil, and various grains. Organisms giving the Voges-Proskauer reaction were shown to be rare in human and animal faeces (21 in 334 or 6.3 %), more common in sewage and pond-water, roof-washings and soil (32 in 65 or 49 %), and still more common in grain (17 in 30 or 57 %); a fair number in the last two groups were, however, chromogenic.

Orr (1908) examined 850 cultures of glucose-fermenters isolated from milk and found that 333 (or 39 %) gave the Voges-Proskauer reaction. The number of cultures fermenting lactose was not recorded.

Houston (1911) examined 532 lactose-fermenters isolated from water and found that 10.3 % from raw river water, 5.3 % from stored river water, and 3.2 % from stored and filtered river water gave the Voges-Proskauer reaction: no evidence of increase of the "V.-P. +" types after storage.

In India, Clemesha (1912) found that *B. lactis aerogenes* ("V.-P. +") was rare in recently polluted waters but became extremely common within a period of 5-15 days after pollution; that this organism became very common in surface waters after rainy seasons, and that *B. cloacae* ("V.-P. +") was the predominant type after dry seasons. He examined 104 samples of human faeces and 1207 cultures, only 4.6 % of these gave the Voges-Proskauer reaction, while from 86 samples of bovine faeces and 1029 cultures this reaction was only given by 13.4 %.

Recently experiments have been carried out by Winslow and Cohen (1918) on the viability of "M.R. +" and "M.R. -" types in water. The average result of 11 experiments showed a relative increase in the "M.R. -" type after nine weeks storage from 46 % to 71 %. These investigators were unable to find a proportion of the "M.R. -" type in gas-producers isolated from unpolluted or stored waters greater than the proportion found in polluted and unstored waters.

To sum up: the results of various investigators show that the lactose-fermenting bacilli can be divided into two main types by the methyl-red or Voges-Proskauer tests, that the "M.R. -" "V.-P. +" type are rare in the faeces of man and animals, are more common in surface water and sewage, and are the predominant type in grain and soil. These findings are in favour of the view that they are either the natural survivors of the lactose-fermenters present in excretal matter, or are derived from soil, or possibly from grain,

and consequently their presence in water and food products is to be regarded as of less sanitary significance than the presence of excretal *B. coli*.

The object of the present investigation was (1) to add confirmation to the results outlined above (no observations based on the methyl-red test have as yet been recorded in this country), (2) to form some idea of the frequency with which the "M.R. —" type is likely to be encountered in water examinations, etc. using bile-salt media, and (3) to throw new light on the sanitary significance of this type in water and food stuffs by noting the presence or absence of *streptococci* in water samples in which they are found<sup>1</sup>.

The following examinations were made:

A. Human and animal faeces.

C. Milk.

B. Cereals, grain and other articles.

D. Water supplies.

#### A. THE EXAMINATION OF HUMAN AND ANIMAL FAECES.

The faeces emulsion in sterile water was in most cases plated direct on lactose bile-salt neutral-red agar. In certain cases, however, it was first cultivated in lactose bile-salt broth and then plated out. Subcultures showing gas-production in lactose broth were cultivated in the glucose di-potassium phosphate medium of Clark and Lubs for five days at 37° C., the culture divided into two portions and tested with methyl-red and for the Voges-Proskauer reaction. With the latter, observations were made 24 hours (or within 24 hours if positive) after the addition of the sodium hydroxide.

The following specimens and cultures were examined and gave results as tabulated below:

Table I.

*Cultures from human and animal faeces.*

Source	No. of Cultures	No. of Individuals	Methyl-red		Voges-Proskauer	
			+	—	+	—
Human faeces	33	11	33	0	0	33
Horse ..	17	8	16	1	0	17
Cow ..	13	7	13	0	0	13
Sheep ..	20	7	20	0	0	20
Rabbit ..	18	6	15	3	3	15
Mouse ..	25	7	22	3	4	21
Cat ..	3	1	3	0	0	3
Guinea-pig faeces	3	1	3	0	0	3
Total	132	48	125	7	7	125
Percentage	—	—	94.7	5.3	5.3	94.7

*Notes on Table I.*

1. The three "M.R. —" strains from rabbit faeces were from the same individual: a tame rabbit fed on oats.

2. The three "M.R. —" "V.-P. +" strains from mouse faeces and the one "M.R. +" "V.-P. —" strain were from three individuals, all wild mice.

3. "M.R. —" strains (not included in the above series) were isolated from two specimens of cow faeces as the ultimate survivors in water after several weeks' storage in the course of some experiments on the relative viability of *B. coli* and *Streptococci* [Savage and Wood (1918)].

<sup>1</sup> It has been shown by Savage and Read (1916) that *streptococci* are to be found in the majority of waters subject to contamination, and by Savage and Wood (1918) that these organisms die out rather more rapidly than *B. coli* and are useful indicators of recent contamination.



The above represent only a limited number of specimens and cultures, but a sufficient number to confirm the findings of other workers, viz. that the "M.R. —" "V.-P. +" type is very rare in human and animal faeces. It has been noted that organisms of the "M.R. —" type frequently gave a less definite reaction on neutral-red lactose bile-salt agar than those of the "M.R. +" type, the paler colonies were accordingly selected when present in order to favour the isolation of the "M.R. —" type as much as possible.

The correlation between the methyl-red and Voges-Proskauer tests was not quite perfect, though very nearly. Some cultures give neutral tints with methyl-red and a positive Voges-Proskauer reaction when peptone other than Witte's is used for the standard medium. Both tests should therefore be applied. The supply of Witte's peptone was exhausted when the above tests were made and Baird and Tatlock's "Bactopeptone" was used and found to be a very good substitute.

Table II.

*Cultural characters of "M.R. —" types isolated from faeces.*

Source	No.	Gas in Lactose	Gas in Saccharose	Litmus milk		Indol	Character of growth on gelatine	Liquefaction of gelatine in 14 days	Voges-Proskauer reaction	Production of capsule
				acid	clot					
Rabbit	4	$\frac{5}{8}$ in.	$1\frac{1}{2}$ in.	+	+	+	...	+	+	+
"	5	$\frac{1}{2}$ "	$1\frac{1}{2}$ "	+	+	+	...	+	+	+
"	6	$\frac{5}{8}$ "	$1\frac{1}{2}$ "	+	+	+	...	+	+	+
Mouse	9	$\frac{5}{8}$ "	$1\frac{1}{2}$ "	+	+	+	...	+	+	+
"	10	$\frac{3}{4}$ "	2 "	+	+	—	...	—	+	+
"	11	$\frac{1}{4}$ "	2 "	+	+	—	...	+	+	+
Cow	1	$\frac{1}{2}$ "	$1\frac{1}{2}$ "	+	+	—	opaque	—	...	+
"	2	$\frac{1}{2}$ "	$\frac{3}{4}$ "	+	+	—	"	—	...	+
"	3	slight	$1\frac{1}{2}$ "	+	+	—	"	—	...	+
"	7	$\frac{1}{2}$ in.	$1\frac{1}{2}$ "	+	+	—	"	—	...	+

*Notes on Table II.*

1. All cultures fermented saccharose and most of them with abundant gas production.
2. All cultures showed capsule formation in milk.
3. Quite a large proportion produced indol.

## B. THE EXAMINATION OF CEREALS, GRAIN AND OTHER ARTICLES.

Sixty specimens were examined, made up as follows:

*Whole grain.* Oats 12, wheat 14, barley 11.

*Flours, etc.* Crushed oats 6, barley flour 2, wheat flour 2, maize flour 4, rice flour 1.

*Other articles.* Straw 2, dried milk 3, egg powder 1, hay 3.

A small quantity was cultivated in lactose bile-salt broth and when acid and gas were produced, plated out on lactose bile-salt agar with neutral-red. In some cases the grains were first allowed to germinate in a sterile moist chamber at 21° C.



Lactose-fermenters were isolated from 20 specimens, from 16 of these only "M.R. —" strains were obtained, while in the remaining four only "M.R. +" strains were found.

The specimens from which the "M.R. —" strains were isolated together with their cultural characters are given in Table III.

Table III.

"M.R. —" strains isolated from cereals, grain, and other articles.

No. of speci- men	Source	No. of culture	Gas in Durham's tube in inches		Litmus milk		Indol	Character of growth on gelatine	Liquefac- tion of gelatine in 14 days	Voges- Proskauer reaction	Produc- tion of capsule in milk
			Lac- tose	Sac- charose	acid	clot					
1	Dried milk	1	$\frac{3}{4}$	$\frac{3}{4}$	+	+	—	opaque*	—†	...	+
		2	$\frac{1}{4}$	$1\frac{1}{4}$	+	+	—	translucent*	—†	...	+
		3	$\frac{1}{8}$	$1\frac{1}{4}$	+	+	—	"	*	—†	+
2	"	4	bubble	$\frac{5}{8}$	+	+	—	"	*	—†	+
		5	$\frac{3}{8}$	$1\frac{1}{4}$	+	+	+	"	*	—†	+
3	Egg powder	6	$\frac{1}{2}$	2	+	+	—	"	—†	+	+
4	Crushed oats	7	$\frac{1}{2}$	$\frac{1}{4}$	+	+	—	"	—†	+	+
		8	bubble	$\frac{3}{8}$	+	+	—	"	*	—†	—
		9	$\frac{1}{4}$	$\frac{1}{2}$	+	+	—	"	—†	+	—
5	Barley flour	10	bubble	$\frac{3}{8}$	+	+	—	"	—	...	...
6	"	11	$\frac{5}{8}$	$1\frac{1}{4}$	+	+	—	"	—	+	—
7	Maize flour	12	1	$\frac{3}{8}$	+	+	+	"	—	+	+
8	"	13	$\frac{1}{8}$	$1\frac{1}{2}$	+	+	—	"	*	—	—
9	Wheat flour	14	$\frac{1}{4}$	$1\frac{3}{4}$	+	+	—	"	+	+	—
10	Straw	15	$\frac{1}{4}$	$\frac{1}{2}$	+	+	—	"	+	...	...
11	Oats	16	$\frac{3}{4}$	$\frac{3}{4}$	+	+	—	"	—	+	—
12	"	17	$\frac{3}{4}$	1	+	+	—	"	—	+	+
13	"	18	$\frac{1}{4}$	...	+	+	—	"	—	...	—
14	Wheat	19	$\frac{3}{8}$	1	+	+	—	"	—	+	+
15	Barley	20	$\frac{1}{2}$	1	+	+ slow	—	"	+	+	+
16	"	21	$\frac{1}{2}$	2							

\* These cultures produced a slight yellow pigment.

† These cultures liquefied gelatine very slowly.

It will be noticed from Table III that the "M.R. —" strains isolated from cereals and grain frequently showed very weak lactose fermentation, less than  $\frac{1}{4}$  inch of gas being produced and this sometimes only after several days. After cultivation on gelatine or in milk at 21° C. this faculty can be revived as the following instances showed:

	Gas produced in lactose broth	
	before cultivation at 21° C.	after cultivation at 21° C.
Barley flour, No. 10	$\frac{1}{16}$ inch	$\frac{1}{2}$ inch
Maize flour, No. 13	$\frac{1}{8}$ "	$\frac{7}{8}$ "
Wheat flour, No. 19	$\frac{1}{4}$ "	over 1 "
Crushed oats, No. 9	$\frac{1}{4}$ "	$\frac{3}{4}$ "

Grain which had been allowed to sprout in a moist chamber at 21° C. usually showed good fermentation of lactose. The "M.R. —" strains also became very numerous under these conditions, and it is quite conceivable

that surface water in the neighbourhood of grain fields might be considerably affected by germinating grain.

A number of cultures liquefied gelatine, but except in three instances too slowly for this test to be of any diagnostic value. A fair number of cultures were chromogenic, but the majority showed no obvious pigment on gelatine slopes. Saccharose was nearly always fermented and frequently with abundant gas production.

Apparently some organisms of the "M.R. —" type possess remarkable viability in grain and flours. The sample of maize flour No. 7 was re-examined after keeping in a sterile bottle for three months and organisms of the "M.R. —" type were again found.

Excluding all cultures giving less than  $\frac{1}{4}$  inch of gas in lactose (but including cultures Nos. 10, 13, 19 and 9), all cultures liquefying gelatine in 14 days or producing pigment, 12 out of the 21 cultures could not certainly be distinguished from *B. coli* of faecal origin except by the methyl-red and Voges-Proskauer tests, though the reaction in litmus lactose broth and on neutral-red lactose agar frequently suggested that they belonged to the "M.R. —" type.

#### C. EXAMINATIONS OF MILK.

Thirty-two samples of milk were examined and 94 lactose-fermenters isolated. Seventeen of these were found to belong to the "M.R. —" type and 77 to the "M.R. +" type.

In the districts from which these samples were obtained the cows are kept and milked in open fields except in the coldest months of the year when they are brought into sheds. It is interesting to compare the "field" samples with the "shed" samples. Only 8.3 % of the former contained "M.R. —" strains while in 28 % of the shed samples this type was found. These results support the suggestion of Rogers, Clark and Evans that the "M.R. —" types found in milk have their origin in grain and straw, but the possibility that the colder weather favoured the predominance of this type must be borne in mind<sup>1</sup>.

The cultural characters of the "M.R. —" strains isolated are given in Table IV.

Table IV.

*Cultural characters of "M.R. —" strains isolated from milk.*

Indol production	Litmus milk acid and clot	Gelatine			Capsule production in milk	Voges- Proskauer reaction
		liquefaction	opaque creamy growth	translucent growth		
10 %	90 %	10 %	40 %	60 %	90 %	100 %

No cultures showed obvious pigment on gelatine slopes.

<sup>1</sup> In a recent paper Rogers, Clark and Lubs (1918) have shown that the "M.R. —" types tend to outgrow the "M.R. +" types when milk is allowed to curdle at 20° C.

## D. THE EXAMINATION OF WATER SUPPLIES.

These comprise a large number of samples from various sources, 200 of which contained lactose-fermenters. The results are given in Tables V and VI, the former were from sources of good repute and the latter from miscellaneous sources.

For the enumeration of *Streptococci* the method of Savage was employed: cultivation in glucose neutral-red broth and examination of hanging drop preparations after 48 hours, in doubtful cases examining stained films with the  $\frac{1}{2}$  inch objective.

Table V.

*Methyl-red negative organisms encountered in the routine examination of 200 water samples containing lactose-fermenting organisms.*

No. of sample	Description of source	Sources of good repute			Identification number of "M.R. -" cultures
		Methyl-red negative type found in	Methyl-red positive type + = present - = absent	<i>Streptococci</i> + = present - = absent	
A. 1620	Well (public supply)	10 c.c.	- 40 c.c.	- 40 c.c.	W. 5
A. 1711	do.	30 "	- 40 "	- 40 "	W. 17
1	Deep well in limestone (public supply)	100 "	- 100 "	- 100 "	W. 19
2	do. do. do.	100 "	- 100 "	- 100 "	W. 20
3	do. do. do.	100 "	+ 100 "	- 100 "	W. 22
x. 1748	Well in field, no source of contamination	30 "	- 40 "	- 40 "	W. 23
4	Deep well in limestone (public supply)	100 "	- 100 "	- 100 "	W. 25
5	do. do. do.	100 "	- 100 "	- 100 "	W. 26
B. 1758	Deep well in limestone	10 "	- 40 "	- 40 "	W. 27
6	Deep well in limestone (public supply)	100 "	- 100 "	- 100 "	W. 29
C. 1809	Deep well sunk through Keuper Marl into Sandstone	10 "	- 40 "	- 40 "	W. 41
7	Deep well in limestone (public supply)	1 "	- 100 "	- 100 "	W. 45, 46, 47
8	do. do. do.	1 "	- 100 "	- 100 "	W. 48, 49, 50
9	do. do. do.	100 "	- 100 "	- 100 "	W. 51
10	do. do. do.	100 "	- 100 "	- 100 "	W. 52
11	do. do. do.	100 "	- 100 "	- 100 "	W. 53
12	do. do. do.	100 "	- 100 "	- 100 "	W. 54
13	do. do. do.	100 "	- 100 "	- 100 "	W. 55
14	do. do. do.	100 "	- 100 "	- 100 "	W. 56
15	do. do. do.	10 "	- 100 "	- 100 "	W. 57, 58
16	do. do. do.	10 "	- 100 "	- 100 "	W. 60, 61
17	do. do. do.	10 "	- 100 "	- 100 "	W. 62, 63
18	do. do. do.	10 "	- 100 "	- 100 "	W. 64, 65
19	do. do. do.	100 "	- 100 "	- 100 "	W. 66
20	do. do. do.	100 "	- 100 "	- 100 "	W. 67
21	do. do. do.	10 "	- 100 "	- 100 "	W. 68
22	do. do. do.	100 "	- 100 "	- 100 "	W. 69
1823	Spring	30 "	- 40 "	- 40 "	W. 70



Table VI.

*Methyl-red negative organisms encountered in the routine examinations of 200 samples of water containing lactose-fermenting organisms.*

Miscellaneous sources.					
No. of sample	Description of source	Methyl-red negative type found in	Methyl-red positive type + = present - = absent	<i>Streptococci</i> + = present - = absent	Identification number of "M.R. -" cultures
1485	Shallow well	30 c.c.	- 40 c.c.	- 40 c.c.	W. 1
1532	do.	10 "	- 40 "	- 40 "	W. 2
1610	do.	$\frac{1}{10}$ "	...	- $\frac{1}{10}$ "	W. 3
1618	Deep well	1 "	...	+ 10 "	W. 4
1622	Shallow well	1 "	...	+ 1 "	W. 6
1642	do.	$\frac{1}{10}$ "	...	+ 1 "	W. 7
1643	do.	10 "	...	- 40 "	W. 8
1652	do.	10 "	...	+ 30 "	W. 9
1658	do.	10 "	...	+ 30 "	W. 10
1694	Deep well	10 "	- 40 "	- 40 "	W. 12
1695	Shallow well	$\frac{1}{10}$ "	+ 1 "	+ 1 "	W. 13
1706	do.	$\frac{1}{10}$ "	+ $\frac{1}{10}$ "	+ 1 "	W. 14
1709	do.	10 "	+ 10 "	+ 1 "	W. 16
1712	do.	10 "	+ 30 "	+ 10 "	W. 18
1734	do.	10 "	+ 30 "	- 40 "	W. 21
1691	Spring	30 "	- 40 "	- 40 "	W. 11
1751	Shallow well	10 "	...	+ 30 "	W. 24
1760	do.	10 "	...	+ 10 "	W. 28
1792	do.	1 "	+ 10 "	+ 30 "	W. 30
1793	do.	30 "	- 40 "	- 40 "	W. 31
1799	Spring	30 "	- 40 "	- 40 "	W. 32
1800	do.	30 "	- 40 "	- 40 "	W. 33
1802	do.	30 "	- 40 "	- 40 "	W. 34
1803	do.	30 "	- 40 "	- 40 "	W. 35
1804	do.	1 "	- 40 "	- 40 "	W. 36, 37, 38
1807	do.	30 "	- 40 "	- 40 "	W. 39
1808	Well	30 "	- 40 "	- 40 "	W. 40
1810	Spring	30 "	- 40 "	- 40 "	W. 42
1813	Well	1 "	...	- 40 "	W. 43
1814	do.	1 "	+ 30 "	+ 30 "	W. 44
1821	Shallow well	10 "	...	+ 10 "	W. 59
1828	do.	1 "	+ 10 "	+ 30 "	W. 73
1836	do.	1 "	+ 10 "	- 40 "	W. 74
1855	do.	$\frac{1}{10}$ "	...	+ 10 "	W. 75
1857	do.	1 "	+ 10 "	+ 30 "	W. 76
1858	do.	$\frac{1}{10}$ "	...	+ $\frac{1}{10}$ "	W. 77
1870	Spring	10 "	...	+ 10 "	W. 78

When *Streptococci* and "M.R. -" strains (the latter in 10 c.c. or less) were both present the evidence of contamination was generally considered sufficiently proven, and the presence of "M.R. +" strains in larger quantities was not as a rule sought. In samples Nos. 1695, 1706, 1709, 1712, 1734, 1792, 1814, 1828, 1836 and 1857 the evidence of contamination, as judged by the presence of both "M.R. -" strains and *Streptococci*, was confirmed by the isolation of "M.R. +" strains from a larger quantity of the sample.

It will be noticed that 29 of the samples (Table V) were from sources of good repute. In only one of these were organisms of the "M.R. +" type also found, and *Streptococci* were found in none.

Thirteen samples from miscellaneous sources contained "M.R. -" strains but no "M.R. +" strains or *Streptococci*.

Out of the total of 66 samples, therefore, in which "M.R. -" strains were found, as many as 41 contained no "M.R. +" strains or *Streptococci*. Judgment of the water was therefore subject to modification in 62 % of these samples, the results suggesting that no recent excretal contamination had occurred.

Of special interest was the occurrence of organisms of the "M.R. -" strain in the deep wells (numbered 1 to 22). This supply which comprises several wells, sunk through limestone some 250-400 feet into underlying sand, has been kept under observation for several years, and the bacteriological results have been very good. Occasionally, in some 15 % of all samples examined, lactose-fermenters have been found, but seldom in less than 100 c.c., and these have nearly always failed to produce indol. In the spring of 1918 lactose-fermenters (all "Lactose + Indol -") were found in a larger proportion of samples, and the application of the methyl-red and Voges-Proskauer tests showed that these with one exception were of the "M.R. -" "V.-P. +" strain. On no occasion were *Streptococci* found. The simultaneous appearance in all the wells, which are several miles apart, is difficult to account for. The time of the year rather suggests some connection with the sowing of grain. Rogers, Clark and Lubs (1918) have recently shown that the majority of "M.R. -" strains derived from grain do not ferment adonitol. Unfortunately a supply of this alcohol was not available and tests could not be made.

#### CULTURAL CHARACTERS OF "M.R. -" STRAINS ISOLATED FROM WATER.

*Lactose litmus peptone.* Eleven cultures (or 18 %) produced  $\frac{1}{4}$  inch or less of gas in the Durham's tube. The litmus indicator frequently showed less acidity than that given by "M.R. +" strains.

*Saccharose.* Only 27 cultures were tested. All fermented saccharose and many with remarkable gas production, the Durham's tube being completely filled in some instances.

*Gelatine.* Liquefaction was observed with six (or 9 %) but only with three was it sufficiently rapid to be of diagnostic value.

None showed obvious pigment.

25 % showed the opaque creamy growth typical of *B. lactis aerogenes*, the rest being more or less translucent.

*Capsule production in milk.* 84 % produced capsules.

*Litmus milk.* 95 % showed acid and clot within a week.

*Indol.* 15 % produced indol in five days.

*Voges-Proskauer reaction.* 92 % gave this reaction.

*Lactose bile-salt neutral-red agar.* Colonies were frequently somewhat paler



than "M.R. +" organisms. The large majority developed mucoid colonies with tendency to become confluent.

English standards as to what is to be considered an excretal type of *B. coli* as distinguished from a coliform organism vary to some extent. Such organisms are required in this laboratory to have the following characters, and it may be taken that this definition would be accepted by most bacteriologists:

*Lactose.* Acid and gas production (a bubble or less than  $\frac{1}{4}$  inch in the Durham's tube excluded)<sup>1</sup>.

*Litmus milk.* Acid and clot production within seven days.

*Gelatine.* No liquefaction within two weeks, and no pigment formation.

*Indol.* Not necessarily produced, but a fairly strong point against its being of recent excretal origin if not produced.

The value of the methyl-red and Voges-Proskauer tests really depends upon the extent to which they are capable of further differentiating organisms *with the above characters* into two groups, one of which is truly excretal and the other non-excretal in origin or very resistant. From this point of view only organisms possessing the above characters need be considered. The following table includes only such organisms:

Table VII.

Material	Number of strains tested	Number of strains			Percentages	
		"M.R.+"	"M.R. -" Indol+	Indol -	"M.R.+"	"M.R. -"
Human faeces	33	33	0	0	100	0
Animal faeces	99	91	4	4	91.9	8.1
Cereals and grain	15	4	1	10	26.5	73.5
Water	231	154	12	65	66.7	33.3
Milk	93	77	2	14	82.5	17.5

The Committee appointed by the Council of the Royal Institute of Public Health in 1914 recommended that in the bacterioscopic examination of waters reports should be based upon the enumeration of "Lactose + Indol +" organisms. If as strict a definition as this be adopted it is evident that only 19 strains from the above series would come under consideration. In judging the purity of a supply from an individual sample, or even from a limited number of samples, however, it is not possible to take so strict a line. In the author's experience a water supply to which an outbreak of typhoid fever was definitely traced yielded only Lactose + Indol - organisms in the first three examinations together with *Streptococci*, subsequent examinations yielding Lactose + Indol + organisms. In the above series of water examinations 13.5 % of the lactose-fermenters did not produce indol and did *not* belong to the "M.R. -" type. Moreover, some of the Lactose + Indol + organisms belong to the "M.R. -" type, Levine (1918) recorded as many as 25 % producing indol.

<sup>1</sup> Some bacteriologists use gelatine shake cultures for observation of lactose fermentation. This is extremely sensitive and cultures producing only a bubble of gas in the Durham's tube would be recorded as positive.



## SUMMARY AND CONCLUSIONS.

1. Investigations by American bacteriologists have shown that the lactose-fermenting (gas-producing) bacilli can be divided into two main types distinguishable by the methyl-red and Voges-Proskauer reactions.

2. The methyl-red — Voges-Proskauer + type are shown to be rare in human and animal faeces, more common in surface water, milk and sewage, and the predominant type in soil and grain, and to be more resistant than the methyl-red + Voges-Proskauer — type.

3. Investigations by the author of this paper confirm their findings as regards human and animal faeces, water, milk and grain. An investigation of the types present in soil is being undertaken and it is hoped to publish an account of this later. Already the "M.R. —" type has been found to predominate in four out of six samples of soil.

4. In the present investigation organisms of the "M.R. —" type were found in 66 samples of water out of a total of 200 containing lactose-fermenters, and in 41 samples containing this type no evidence of recent contamination could be adduced by the search for organisms of the "M.R. +" type or *Streptococci*. Judgment of the water was therefore liable to modification in 20 % of these samples by the recognition of this type.

5. Twenty-nine out of the 66 samples containing organisms of the "M.R. —" type were from sources of good repute, most of them from public supplies.

6. The presence of lactose-fermenters of the "M.R. —" "V.-P. +" type must be regarded with considerably less disfavour than the presence of "M.R. +" "V.-P. —" organisms, and the application of tests for the recognition of these types is important. It is suggested that these tests should be included in all routine examinations of water and food products.

In conclusion I have pleasure in acknowledging my indebtedness to Dr W. G. Savage for calling my attention to the researches of American bacteriologists and for many valuable suggestions he has made in connection with this work.

## REFERENCES.

- BURTON and RETTGER (1917). *Journ. Infect. Dis.* XXI. 162.  
CLARK and LUBS (1915). *Ibid.* XVII. 160–173.  
CLEMESH (1912). *The Bacteriology of Surface Waters in the Tropics*, p. 106 and Table II c.  
HOUSTON (1911). *7th Research Report, Metropolitan Water Board*, pp. 30–32.  
JOHNSON (1916). *Journ. Bacteriology*, I. 96.  
LEVINE (1915). *Journ. Infect. Dis.* XVIII. 358.  
—— (1918). *Journ. Bacteriology*, III. 253–276.  
LEVINE, WELDIN and JOHNSON (1917). *Journ. Infect. Dis.* XXI. 39.  
MACCONKEY (1906). *Journ. Hygiene*, VI. No. 3.  
—— (1909). *Ibid.* IX. No. 1.  
MYRTLE GREENFIELD (1916). *Journ. Infect. Dis.* XIX. 647.  
ORR (1908). *Report on an investigation as to the contamination of milk*.

ROGERS (1916). *Journ. Bacteriology*, I. 82.

ROGERS, CLARK and DAVIS (1914). *Journ. Infect. Dis.* XIV. 411-475.

ROGERS, CLARK and EVANS (1914). *Ibid.* XV. 100-123.

—— (1915). *Ibid.* XVII. 137-159.

ROGERS, CLARK and LUBS (1916). *Journ. Bacteriology*, I. 82.

—— (1918). *Ibid.* III. 231-252.

SAVAGE and READ (1916). *Journ. Hygiene*, XV. No. 3.

SAVAGE and WOOD (1918). *Ibid.* XVI. No. 3.

WINSLOW and COHEN (1918). *Journ. Infect. Dis.* XXIII. 82-101.

# THE CULTIVATION OF *SPIROCHAETA ICTEROHAEMORRHAGIAE* AND THE PRODUCTION OF A THERAPEUTIC ANTI-SPIROCHAETAL SERUM.

BY A. STANLEY GRIFFITH, M.D.

(*Report to the Medical Research Committee.*)

(*From the Field Laboratories, University of Cambridge.*)

## INTRODUCTION.

THE Japanese investigators (Inada and Ido) who discovered the *Spirochaeta icterohaemorrhagiae*, the cause of infectious jaundice, showed that the serum of patients who had recovered from the disease contained immune substances which were capable of destroying the spirochaetes in the blood and tissues of an experimentally infected guinea-pig. They found that if the serum were injected into the guinea-pig prior to the appearance of icterus the disease was inhibited in all cases. The same result was obtained with the serum of immunised goats.

On the basis of this work Inada and Ido advanced the hypothesis that serum therapy should be effective in the human disease.

In the treatment of human cases they tried in the first instance the serum of recovered human cases and later the serum of actively immunised horses.

As the result of these first trials they came to the conclusion that the administration of the immune serum had a beneficial effect in cases of the disease.

Cases of jaundice with the clinical course of infectious jaundice were first observed among the troops on the Western front in the summer and autumn of 1915 but it was not until 1916, following the publication of the Japanese work, that *Spirochaeta icterohaemorrhagiae* was demonstrated in the blood and urine of the affected persons (Stokes and Ryle, 1916). The spirochaete was also found by Adrian Stokes (1917) in rats captured in the trenches where the cases of jaundice had originated.

In view of the favourable reports by the Japanese of the serum treatment of infectious jaundice and of the possibility of further cases arising among the British troops, seeing that the parasite was being harboured by the trench rat, it was decided by the Medical Research Committee to supply a curative serum for the treatment of cases of the disease.

The work of preparing the serum was entrusted to me and was begun in February 1917.



I am indebted for my original material to Dr J. MacIntosh who sent me a portion of the liver of a guinea-pig which had died of experimental spirochaetosis. The strain of spirochaete used was that known as the Belgian strain and had been originally obtained by Captain Adrian Stokes from a case of infectious jaundice.

This strain had not at that time been brought into artificial cultivation and it was decided after discussing methods with Dr H. H. Dale to begin the immunisation of a horse with tissue spirochaetes and to continue with culture if cultivation experiments were successful.

My work on the subject therefore can be described under two heads dealing respectively with (a) the immunisation of horses with the spirochaete and (b) the artificial cultivation of the spirochaete.

It will be convenient to record first the cultivation experiments.

#### BIOLOGICAL CHARACTERISTICS.

##### ARTIFICIAL CULTIVATION OF *SPIROCHAETA ICTERHAEMORRHAGIAE*.

The Japanese observers, Inada and Ido, succeeded in cultivating *Spirochaeta icterohaemorrhagiae* by the method of Noguchi for the culture of the spirochaete of recurrent fever. I tried this method with various kinds of animal sera and human ascites fluid to which had been added, as recommended by Inada and Ido, guinea-pig kidney instead of rabbit kidney. The media were inoculated with pieces of the liver of an infected guinea-pig and were incubated at 37° C. and 25° C. both under aerobic and anaerobic conditions.

My experiments with these fluid media, like those of Adrian Stokes with similar media, were unsuccessful.

I then tried diluted bovine serum (serum 1 part and physiological salt solution 2 parts) which had been heated to 70° C. until it had become slightly viscous and I inoculated the tubes with the heart blood as well as with fragments of the liver of an infected guinea-pig. In this medium incubated at 25° C. a growth of the spirochaete was obtained at the first attempt and I was able subsequently to transmit the organism through several generations. Shortly after obtaining the first culture I had the opportunity, through the courtesy of the Secretary of the Medical Research Committee, of reading an advance proof of Noguchi's paper (1917) in which he reported the cultivation of the Belgian as well as the French and American strains of the spirochaete.

Noguchi grew the spirochaete in a fluid medium in which there were loose strands of fibrin produced by adding a small quantity of citrate plasma to the diluted or undiluted serum of a suitable animal. He recommended for routine use two media which had given equally satisfactory results:

(a) Rabbit serum (1 part) + Ringer's solution or 0.9 per cent. sodium chloride solution (3 parts) + citrate rabbit plasma (0.5 part) covered with a thin layer of sterile paraffin oil; (b) the same, except for the use of 0.5 to 1.0 part of neutral or slightly alkaline

agar (2 per cent.) which should be added while in a liquid state and quite hot (60–65° C.) in order to get a uniform mixture of the agar.

I was able to cultivate the spirochaete in these media but I obtained more luxuriant initial cultures in diluted serum which had been previously heated until it had become viscous or semi-gelatinous. This kind of medium was therefore adopted for the routine primary cultivation of the spirochaete.

The sera used were those of the horse, rabbit and adult cattle. Of these bovine serum has given the most consistently good results and has been most frequently employed. Bovine serum was also found by Martin, Pettit and Vaudremier (1917) to be very suitable for the cultivation of the spirochaete. Horse serum in a semi-gelatinous state proved less favourable for the growth of the spirochaete than semi-gelatinous bovine or rabbit serum, but horse serum unheated and mixed with 0.5 per cent. agar was found to be not inferior in nutritive value to bovine and rabbit serum.

Very satisfactory results have also been obtained with a medium composed of 1 part of the citrated blood of the horse, cow or rabbit mixed with 1 or 2 parts of physiological saline and heated until semi-gelatinous. In the horse blood medium the spirochaete sometimes grew extremely luxuriantly and one strain was transmitted through many subcultures in this medium. Ito and Matsuzaki (1916) recommended the use of a blood gelatin or a blood agar medium.

Noguchi (1918), in a recent paper, has recorded the results of his investigations of the value of different animal sera for the cultivation of the spirochaete. He found that the cultural value of different animal sera varies considerably. It is entirely absent from the sera of the rat and the pig. The sera of the rabbit, horse and goat are better suited for the growth of the organism than those of the guinea-pig, sheep, donkey or calf. Human serum is suitable but not ascites fluid. Fresh and heated emulsions of organs and the white and yolk of hens' eggs have no cultural value. (I also tried an egg medium, and, like Noguchi, failed to get a growth of the spirochaete.)

Noguchi found further that the nutritive value of serum is considerably reduced by heating to 60° C. for 30 minutes. My few comparative observations bearing on this latter point support Noguchi's finding. It has been observed that the addition of a small quantity of the citrated blood of a normal guinea-pig increases the nutritive value of a heated serum medium, thus indicating that the fresh blood restored to the medium something which was lost in the process of heating. This enrichment was specially noted with horse and rabbit serum. Bovine serum on the other hand, especially when of a golden yellow colour in the fluid state, showed little diminution in nutritive value after heating and produced luxuriant subcultures without any addition of fresh blood. With guinea-pig blood added semi-gelatinous bovine serum is in my experience an excellent medium for the cultivation of the spirochaete and by its use I have kept a strain of the organism in artificial cultivation for more than 18 months.



For raising initial cultures from an infected guinea-pig the citrated heart blood is the most convenient and suitable material. Liver and even kidney may be used but the cultures are more liable to become contaminated with other organisms than when blood is the material employed. Subcultures are made by transferring with a sterile pipette a few drops from the top of the medium to the surface of the fresh medium. All media are covered with a thin layer of sterile paraffin oil.

The spirochaete is an obligatory aerobe and grows best as Noguchi has also observed in the upper centimetre of the medium. When the medium is gelatinous spirochaetes are found only in small numbers in the middle and often not at all in the deep parts of the tube. In fluid media on the other hand there is wider distribution of the organism throughout the medium, possibly because oxygen, as Noguchi suggests, is able to penetrate more deeply into a fluid than into a semi-solid medium.

In semi-gelatinous media the spirochaetes are at first unevenly distributed in the upper layer. While some areas are colonised by enormous numbers of the organism others contain only moderate numbers and others again very few or none.

The spirochaete has been cultivated by me at two temperatures only, namely at 37° C. and 25° C. It is stated that it also grows at lower temperatures, down to 10° C.

Multiplication is as a rule more rapid at 37° C. than at 25° C. but sometimes, in primary cultures, growth has been as rapid at 25° C. as at 37° C.

After two days' incubation at 37° C. (primary culture, semi-gelatinous serum medium) spirochaetes may be found in swarms in the upper centimetre of the medium. At the end of a week they are less numerous and degenerate forms are seen. In a fortnight they have diminished in numbers very considerably and there are many granules, the remains of degenerated spirochaetes. In the fourth week they have often disappeared completely from the medium which is no longer capable of infecting the guinea-pig. In order therefore to maintain a culture of the spirochaete at 37° C., subcultures should be made at short intervals. I did not however in my early experiments succeed in transmitting the spirochaete through more than five generations at this temperature and this result supported the statement of Inada and Ido (1916) that 37° C. is unfavourable for the cultivation of the organism. Recently I have had better success at this temperature, using a strain of the spirochaete which had been in cultivation for more than a year at 25° C.; this strain has been for several generations and is now growing luxuriantly at 37° C.

At 25° C. growth is in general not so rapid as at 37° C. but the organism retains its vitality for a longer period and can be subcultured through many generations. In subcultures at 25° C. spirochaetes do not as a rule become numerous until the second or the third week. When the medium is semi-gelatinous the spirochaetes begin after a time (two to three months) to degenerate and gradually to disappear while in more fluid media, though they



may diminish in number, they persist and remain viable for indefinite periods.

The Japanese observers, Inada and Ido (1916), stated that the life of a culture is variable.

The first generation lived mostly from three to six weeks, the longest period observed being 55 days and the shortest 17 days. The life of the 2nd and 3rd generation is somewhat shorter than that of the first generation. The best time for transferring the culture from one tube to another is when multiplication is going on rapidly as indicated by examination of the fluid every two or three days.

My observations, while confirming the statement that the length of life of individual cultures varies, show that in a suitable medium the organism may live for very long periods not only in primary but also in secondary cultures.

Transference of the cultures have usually been made at intervals of from two to six weeks but subcultures up to 16 weeks old have grown luxuriantly in fresh tubes of medium. In fact there seems to be no limit to the age of a culture for successful subcultivation; if spirochaetes are present in the medium they will grow in a suitable new medium no matter what their age.

In order to ascertain how long a culture of the spirochaete would remain viable the first primary culture has been preserved (at 25° C.) and has been tested at intervals, the last test being made when the culture was 15 months old. On every occasion the spirochaetes were found to be capable of growing vigorously and luxuriantly in subculture.

No final statement therefore can be made at present as to the maximum duration of life of the spirochaete outside the body in a favourable medium. The primary culture is being preserved and will be tested again after a more prolonged interval.

#### PATHOGENICITY OF CULTURES.

Young recently isolated cultures of the spirochaete produce the same morbid effects in the guinea-pig as tissue spirochaetes. The pathogenicity of the cultivated spirochaete however appears quickly to be lost. A culture grown at 37° C., which, when a fortnight old produced typical spirochaetosis in a guinea-pig, lost its virulence within the next fortnight (two experiments). In another experiment with spirochaetes grown at 25° C., a 14 days' old primary culture produced fatal haemorrhagic jaundice while the same culture when 3½ and 4 months old was completely non-pathogenic. A 14 days' old subculture from the four months' old primary culture was also devoid of virulence for the guinea-pig.

The guinea-pigs which failed to develop spirochaetosis after the injection of living cultures were after intervals of a month inoculated with emulsion of liver from a fatally infected guinea-pig. The guinea-pigs were entirely unaffected though control guinea-pigs receiving the same amounts of emulsion died in three to four days of acute spirochaetosis.

The above observations show that the virulence of the spirochaete for the guinea-pig is soon lost in culture and that the attenuated cultures of the organism are capable of inducing an active immunity in guinea-pigs.

Attempts to raise the virulence of the attenuated cultures by passage through the bodies of very young guinea-pigs, rats, mice and toads have not so far been successful.

The rats and mice were killed two to four weeks after intraperitoneal injection of the cultures and their kidneys were emulsified and injected into guinea-pigs. No spirochaetes were found in the emulsions, or in smear preparations of the spleen, liver and blood, and the guinea-pigs remained unaffected.

Toads (three experiments) were used with a view to ascertaining whether the spirochaete is capable of multiplying in the bodies of cold blooded animals. No spirochaetes were found in smear preparations of their organs, heart blood or subcutaneous lymph 14 and 28 days after inoculation.

#### THE MORPHOLOGY OF THE SPIROCHAETE AND THE PATHOGENIC EFFECTS IN THE GUINEA-PIG.

My observations on the morphology of *Spirochaeta icterohaemorrhagiae* and on the gross pathological appearances of experimental spirochaetosis in the guinea-pig agree with those of other workers (Noguchi, 1918; Stokes and others, 1917; Inada and others, 1916) and I have nothing new to add to what has already been published in these connections.

#### THE IMMUNISATION OF HORSES.

##### *Horse 1.*

On 6 February, 1917, the immunisation of a horse was begun. As stated in the introduction it was decided to use as antigen in the first place emulsions of the livers of guinea-pigs which had died of experimental spirochaetosis and to continue with cultures if cultivation experiments were successful.

During the first four weeks the horse received seven injections of liver emulsions which had been sterilised by heat (at first 55° C. and subsequently 50° C.) or by the action of 0.5 per cent. phenol. The injections were made intramuscularly, the first dose being 5 c.c. of a thick emulsion, the seventh 20 c.c. containing the emulsifiable tissue of the livers of two young guinea-pigs.

After the latter injection the serum was found by Dr J. MacIntosh to have definite agglutinative and lytic action on living spirochaetes and it was considered therefore that the administration of the living organism might safely be begun.

The first dose of the living virus was 5 c.c. of liver emulsion rich in spirochaetes. The doses were thereafter gradually increased up to 25 c.c. containing the emulsifiable tissue of the livers of three guinea-pigs. As cultures of the spirochaete were now available the next dose (or tenth dose of living



virus) was partly liver emulsion and partly culture. Unfortunately only one guinea-pig was available and, as the cultures were only moderately luxuriant, this dose of antigen was therefore less rich in spirochaetes than the previous ones.

With a view to the speedy attainment of a high titred serum it was decided at this stage to continue the immunisation by the intravenous inoculation of cultures.

20 c.c. of a fluid culture containing numerous spirochaetes were accordingly injected into the jugular vein on June 15. Shortly after the injection the horse began to show signs of distress; then clonic contractions of the muscles set in and the horse fell down unconscious; respiration continued for a few moments when death ensued.

#### TESTS OF THE POTENCY OF THE SERUM.

The titre of the serum was determined at various stages of the immunisation by ascertaining the quantity of serum which would suffice to protect a guinea-pig weighing from 200 to 300 grams from a fatal dose of spirochaetes.

The test dose of virus was 1.0 c.c. of an emulsion of guinea-pig liver rich in spirochaetes. The doses were not standardised but the emulsions used were approximately of equal density. Though there was no doubt considerable variation in the numbers of spirochaetes injected in the different sets of experiments this variation did not appear to affect materially the comparative value of the tests.

The serum was injected a few minutes after the virus. Both virus and serum were injected intraperitoneally.

Three samples of the serum were tested. The first taken after about two months' immunisation gave a titre of 0.25 c.c., *i.e.* this amount of serum completely protected a guinea-pig from the test dose of spirochaetes. The second sample (May 11) had a titre of 0.1 c.c., while the third (June 14) was rather less potent than the second, 0.1 c.c. failing to protect the guinea-pig from death though it considerably prolonged the duration of life. The fall in the potency of the serum on June 14 is attributable to an insufficiency of antigen in the preceding dose (see above).

#### *Horse 2.*

The immunisation of a second horse was begun on June 18, 1917. During the first 18 days the horse received seven increasing doses of killed spirochaetes contained either in liver emulsions or in culture fluids.

A sample of serum taken five days after the seventh injection and 23 days after the beginning of the immunisation was tested for immune bodies.

#### *First potency test.*

Number of guinea-pig	Quantity of serum	Duration of life of guinea-pigs	Result
2 controls	—	Both dead, 4 days	Typical spirochaetosis
2469	1 c.c.	Died, 18 days	No haemorrhages or jaundice
2470	0.5 c.c.	Died, 15 days	Typical spirochaetosis
2471	0.25 c.c.	Died, 11 days	do.



This test showed that 1 c.c. of the serum was capable of protecting a guinea-pig from a dose of spirochaetes which killed controls in four days while smaller amounts of the serum prolonged the lives of the guinea-pigs.

The immunisation of the horse was continued with living spirochaetes derived either from cultures or from the livers of fatally infected guinea-pigs.

A second test of the serum was made after the sixth dose of living virus, 54 days from the beginning of immunisation.

*Second potency test.*

2 controls	—	Both dead, 4 days	Typical spirochaetosis
2482	0.5 c.c.	Died, 31 days	No sign of disease
2483	0.25 c.c.	Survived	
2484	0.125 c.c.	do.	

Another guinea-pig receiving 1 c.c. of the serum two days after the test dose of emulsion also survived.

This test showed that the serum had at least eight times the potency of the first sample.

As the doses increased in size it was found more convenient to use as principal antigen liver emulsion than culture. Liver emulsions contain enormous numbers of spirochaetes and to obtain approximately equal numbers in cultures would have required very considerable quantities of nutrient media. Whenever cultures were available however these were administered along with the emulsions. The largest dose of culture injected at one time was 30 cubic centimetres.

On September 18, eight days after the nineteenth dose of antigen and three months from the beginning of immunisation, the horse was bled 6 litres.

*Third potency test.*

Three sets of experiments were carried out with this serum. In the first two the smallest quantity of serum used was 0.05 c.c. and this in the two instances in which it was given afforded complete protection from a dose of spirochaetes which killed controls in from six to nine days.

A third series was done in order to ascertain the minimum quantity of serum which would protect, and the result is set out in the following table:

3 controls	—	All dead, 4 days	Typical spirochaetosis
2543	0.05 c.c.	Died, 104 days	No disease
2544	0.025 c.c.	Survived	—
2545	0.0125 c.c.	Died, 9 days	Typical spirochaetosis

On November 24, eight days after the 25th dose, which was 66 c.c. of an emulsion made from the livers of five guinea-pigs, the horse was bled 8 litres.

*Fourth potency test.*

2 controls	—	Died in 5 days	Typical spirochaetosis
2580	0.0125 c.c.	Survived	
2581	0.01 c.c.	Died, 6 days	No jaundice or haemorrhages
2582	0.01 c.c.*	Died, 5 days	
2583	0.006 c.c.*	Died, 6 days	No jaundice, slight haemorrhages
2584	0.005 c.c.*	Died, 6 days	Typical spirochaetosis

\* Incubated with test dose 45 minutes.

This test showed that since September 18 the serum had increased considerably in potency. 0.0125 c.c. now protected a guinea-pig completely from a dose which was fatal to controls in five days, while 0.01 c.c. was able apparently to destroy the spirochaetes in the test dose, though unable to save the lives of the guinea-pigs (microscopical examination of the liver of one of these latter guinea-pigs failed to reveal a spirochaete).

The immunisation of the horse was continued until the middle of March 1918, by which time 33 immunising doses had been given. On March 28 the animal was bled again. The potency of this batch of serum has not yet been tested because of the loss of the strain of spirochaetes which was being passed through guinea-pigs. Since January 1917 the strain had been passed from guinea-pig to guinea-pig until March 1918 when owing to a temporary shortage of young guinea-pigs adult guinea-pigs had to be used. The first of these died in seven days of spirochaetosis, but material from it failed to produce the disease in a second adult guinea-pig.

The first batch of serum sent out for the treatment of cases was from the first horse taken before the last and fatal dose of antigen. The titre of this serum was not very high (between 0.2 and 0.1 c.c.) but it was higher than that of the serum (titre 0.5 c.c.) used by the Japanese in their first series of cases. It was therefore supplied for trial in any case which might arise before a more potent serum became available.

The therapeutic results obtained by the Japanese workers (Inada, Ido, Hoki, Ito and Wani, 1916) with the low titred serum were very favourable. The serum considerably reduced the mortality rate and was found to be capable of destroying completely the spirochaetes in the circulating blood. Moreover the treatment promoted the development of antibodies and reduced the number of spirochaetes in the organs. At first they injected 10 c.c. of the serum into the subcutaneous tissues daily for three days but experience showed that this dosage was ineffectual. They therefore increased it gradually until finally they injected 60 c.c. in 24 hours. Later on they injected the serum intravenously and they found that this method far exceeded in potency subcutaneous injection. In a recent publication (1918) these observers give the results of the intravenous serotherapy of 41 cases of infectious jaundice. The spirochaetocidal titre of the sera used in this series was 0.01 and 0.03 c.c.

As a rule they injected 60 c.c. intravenously irrespective of severity of illness. Sometimes the entire quantity was given within 24 hours; at other times 40 c.c. in one day and 20 c.c. on the following day, or 20 c.c. on three successive days.

The action of the immune serum is spirochaetocidal and spirochaetolytic and best results are obtained when injections are made at an early stage of the disease. They found that intravenous injections are effective up to the fifth and sixth days of illness.

The total mortality figures, particularly of the severer cases, was considerably lower in the serum treated cases (intravenous and subcutaneous) than in the non-serum treated cases.

When the serum is administered early the disease appears to assume a milder form. The immune serum shortens the duration of the illness and has also a definitely beneficial influence upon haemorrhages, heart rhythm and suppurative processes.

The results obtained in this series led the authors to the conclusion that a titre of 0.01 c.c. suffices for the efficient serotherapy of infectious jaundice.

The November 24 serum from my second horse attained to this standard and should therefore be as effective in the treatment of the disease as the Japanese serum.

I have not yet any results to record of the therapeutic use of the serum.

#### REFERENCES.

- DAWSON, B., HUME, W. E. and BEDSON, S. P. (15. ix. 1917). *Brit. Med. Journ.* II. 345.  
INADA, R., IDO, Y., HOKI, R., KANEKO, R., and ITO, H. (1916). *Journ. Exper. Med.* XXIII. 377.  
INADA, R., IDO, Y., HOKI, R., ITO, H. and WANI (1916). *Ibid.* XXIV. 485.  
— (1918). *Ibid.* XXVII. 283.  
ITO, T. and MATSUZAKI, H. (1916). *Ibid.* XXIII. 557.  
MARTIN, L., PETTIT, A., and VAUDREMER, A. (1917). *Compt. rend. Soc. biol.* LXXX. 197.  
NOGUCHI, H. (1917). *Journ. Exper. Med.* XXV. 755.  
— (1918). *Ibid.* XXVII. 593.  
— (1918). *Ibid.* XXVII. 575.  
STOKES, A. and RYLE, J. A. (1916). *Journ. Roy. Army Med. Corps*, XXVII. 286.  
STOKES, A., RYLE, J. A. and TYTLER, W. H. (1917). *Lancet*, I. 142.



## THE ATYPICAL DYSENTERY BACILLI.

By T. J. MACKIE, M.B., D.P.H.,

*Professor of Bacteriology, University of Cape Town.*

As there appears to be a considerable amount of uncertainty (as evidenced by recent writings on this subject) regarding the biological relationships and the etiological significance of the so-called "atypical *B. dysenteriae*," this short communication is intended as a brief résumé of conclusions arrived at in the course of an extended investigation of dysentery bacilli in Egypt during 1916 and 1917. It is to be noted that the results recorded here were obtained from the examination of cases of dysentery observed in the earliest stages and frequently followed into convalescence. Such experience has shown that conclusions derived solely from an investigation of the later stages of the disease, *e.g.* convalescents examined after arrival from over-seas in the United Kingdom, throw little light on the problem of the bacterial etiology of the disease as a whole. In a paper by Thomson and Mackie (1917)<sup>1</sup> on the clinical and laboratory study of dysentery in Egypt a number of atypical dysentery strains were described, but at the time this communication was submitted for publication my observations were not sufficiently complete to make any general statements regarding organisms of this type and no attempt was made to classify them.

When atypical and inagglutinable strains were first encountered in the course of laboratory examination of dysentery cases, the tendency was to disregard their occurrence and only the classical types were accepted for diagnostic purposes. As time went on, however, and as the proportion of cases from which classical dysentery bacilli could be isolated was unexpectedly low, attention was once more directed to these atypical varieties especially when they occurred in large numbers in the excreta during the earlier phase of an acute case in which amoebae were absent. Some of them corresponded in all their cultural reactions to the Shiga or Flexner-Y types, but failed to react to specific agglutinating sera. These came to be designated "inagglutinable *B. dysenteriae* Shiga or Flexner-Y." Only a few of these became agglutinable after repeated subcultures on artificial media, when tested two to three months after isolation, and some which in primary culture appeared to correspond to this designation rapidly underwent spontaneous mutation

<sup>1</sup> *Journ. Royal Army Med. Corps*, xxviii. 403. The present author is personally responsible for the bacteriological notes in this paper.

and displayed fermentative characters which clearly differentiated them from the classical types. The close biological similarity to the group of classical dysentery bacilli was nevertheless very striking, and from their constant occurrence in a certain proportion of all the acute dysenteries and their characteristic toxic effects on animals (*v. infra*), they came to be accepted as dysentery producing organisms.

#### CLINICAL TYPES OF DYSENTERIC INFECTIONS.

Dysenteric infections displayed a considerable diversity in their manifestations and varied from the typical acute attack of dysentery with the characteristic blood and mucus stools, to a simple diarrhoeal illness without obvious blood or mucus discharges. Generally speaking the latter type was characterised by the presence of more or less abundant cellular exudate evident on microscopic examination of the stools. Thus two types of infection might be distinguished: (1) an acute type with blood and mucus in the stools, (2) a milder type with fluid stools containing abundant cellular exudate but without blood or mucus. This distinction is of importance, as will be seen later, in determining the relationship of the atypical dysentery bacilli to the classical organisms as regards their pathogenic effects. The typical dysentery bacilli (Shiga and Flexner-Y types) were found to be associated with both the severe and the milder infections, but the great majority of the Shiga infections were of the acute type while, in the case of the Flexner-Y infections, the proportion of acute cases was smaller and the number of ordinary diarrhoeal cases larger as compared with the Shiga infections (see also below).

#### *B. DYSENTERIAE* SHIGA STRAINS.

The Shiga strains isolated invariably corresponded in their cultural and biochemical reactions to the classical type and showed a specific agglutination reaction (*i.e.* to end titre) with a Shiga agglutinating serum. Only a few strains which corresponded in cultural reactions to the Shiga type and did not agglutinate in primary culture, became agglutinable after repeated subculture. No non-mannite fermenting strains, which resembled the Shiga type in most of their cultural characters, but differed as regards the fermentation of maltose or the production of indol, ever agglutinated with the Shiga serum. I originally thought that such strains and also the so-called inagglutinable *B. Shiga* might be "variants" from the classical type, and this was noted in the original paper by Thomson and myself. I ultimately classified these along with the atypical dysentery bacilli.

#### *B. DYSENTERIAE* FLEXNER-Y STRAINS.

In the ultimate identification of organisms of the Flexner-Y type, the Lister Institute Y serum<sup>1</sup> was used: and it may be said that the strains of

<sup>1</sup> Prepared with the original strain of Hiss and Russell, see Chick, *Lancet*, April 22, 1916.



this group invariably corresponded in their cultural characters to the classical types and showed a *specific* agglutination reaction with this serum to end titre. Saccharose fermenting strains which reacted to specific serum have been described by other observers, *e.g.* Martin<sup>1</sup>, Glynn and others<sup>2</sup>, but I invariably found that the Flexner-Y strains failed to ferment saccharose and lactose, and that mannite fermenting strains which after some days' incubation fermented saccharose or lactose were not agglutinated by the Y serum, accordingly they were classified with the atypical group.

A "Flexner Serum" (R.A.M. College) was also used in parallel series in the agglutination tests of a considerable number of mannite fermenters and only a small proportion of these reacted to it even in low titres.

I prepared a high titre agglutinating serum to a strain which reacted specifically to the Lister Y serum, but not to the Flexner serum, and found that this serum only agglutinated a small number of strains which were agglutinated to end titre by the Lister Y serum, and none of these reacted to the Flexner serum. This may be represented as follows:

	Lister Y serum	Flexner R.A.M. College serum	Serum to a strain agglutinated by Y serum
A. Majority of strains of Flexner-Y group ... ..	+	-	-
B. A small number of strains of Flexner-Y group ... ..	+	+	-
C. A small number of strains of Flexner-Y group (not the same strains as are included in group B)	+	-	+

(+) indicates agglutination up to "end titre" of the serum.

This subject requires further investigation, but it would appear that the Flexner-Y group includes perhaps a number of species and that the strain with which the Lister Y serum is prepared represents antigenic properties common to practically the whole group. A strain which was agglutinated by the Flexner serum was invariably agglutinated by the Y serum. Chick<sup>3</sup> has also noted the more restricted degree of specificity shown by "Flexner" sera for mannite fermenting strains as compared with the action of Y serum. It is further noteworthy that some strains of "Shiga" are agglutinated up to end titre by this "Y" serum. Only a small number of strains found to be inagglutinable in primary culture became agglutinable after subculture.

With regard to the agglutination of organisms of the dysentery group, it is to be noted, that, as contrasted with the typhoid group, sedimentation occurs comparatively slowly and the clumps are much smaller and the sediment is granular rather than flocculent. The results recorded here were obtained by incubating organisms (suspensions of 24 hour agar-cultures) and serum for two hours at 37° C. and then allowing the tubes to stand overnight at room-temperature.

<sup>1</sup> Martin, *Brit. Med. Journ.* i. April 14, 1917.

<sup>2</sup> Glynn, Berridge, Foley, Preece and Robinson, *Report to Medical Research Committee*, December, 1917.

<sup>3</sup> *Lancet*, April 22, 1916.



THE "ATYPICAL *B. DYSENTERIAE*."

This group may now be defined as (1) Gram-negative, non-motile bacilli, not liquefying gelatin, always fermenting glucose without gas production, (2) different strains varying as regards the fermentation of lactose, dulcitol, saccharose, mannitol, maltose (but never producing gas in any case) and the formation of indol from peptone, and (3) not agglutinated by a Y, Flexner, or Shiga serum. The cultural reactions when once acquired are all stable, as determined by repeated examination of strains. The reactions of some of the types met with are shown in Table I.

Table I.

Atypical Dysentery Bacilli.

Atypical <i>B. dysenteriae</i>	Motility	Glucose	Lactose	Dulcitol	Saccharose	Mannitol	Maltose	Indol	Gelatin	
No. 1	-	±	-	-	-	-	-	-	-	{ Corresponds to <i>B. dysenteriae</i> Shiga but not agglutinable by specific anti-Shiga serum
2	-	±	-	-	-	-	-	+	-	
3	-	±	-	-	-	-	±	-	-	
4	-	±	-	-	-	-	±	+	-	
5	-	±	-	-	-	±	-	-	-	{ Correspond to <i>B. dysenteriae</i> Flexner-Y but not agglutinated by specific anti-Y serum
6	-	±	-	-	-	±	-	+	-	
7	-	±	-	-	-	±	±	-	-	
8	-	±	-	-	-	±	±	+	-	
12	-	±	-	-	±	-	±	+	-	{ Corresponds to <i>B. dysenteriae</i> Strong
16	-	±	-	-	±	±	±	+	-	
17	-	±	-	±	-	-	-	-	-	
22	-	±	-	±	-	±	-	+	-	
24	-	±	-	±	-	±	±	+	-	
32	-	±	-	±	±	±	±	+	-	
40	-	±	±	-	-	±	±	+	-	
48	-	±	±	-	±	±	±	+	-	
56	-	±	±	±	-	±	±	+	-	
64	-	±	±	±	±	±	±	+	-	

± = acid no gas.

These reactions when once acquired are all stable as determined by repeated examination of strains.

The numerical classification is based on the various possible combinations of cultural reactions.

All the various types were proved to be extremely virulent by intravenous or intraperitoneal injection of rabbits, producing a characteristic haemorrhagic enteritis, which was most marked in or limited to the small intestine. In fact, the virulence experiments demonstrated a highly selective toxic action on the mucosa of the small intestine (affecting the stomach to a less degree), which on autopsy was found to be intensely inflamed with massive haemorrhages in the tissue, and the lumen of the intestine was usually distended with blood-stained muco-purulent material loaded with cellular exudate and

masses of exfoliated epithelium. In the case of intraperitoneal injections, often there was little reaction in the peritoneum, but the characteristic effect on the intestine was distinct. Cultures from the intestinal contents usually yielded an almost pure growth of the particular organism. These results were obtained as a rule by injecting  $\frac{1}{8}$  or  $\frac{1}{4}$  of an agar slope culture in saline and the animals died in about 24 hours. Some strains, however, including a number of atypical varieties, exhibited a much higher virulence and the intravenous injection of  $\frac{1}{40}$  of a 24 hours' agar slope culture produced the characteristic haemorrhagic enteritis. Comparative tests with a recently isolated Shiga and a No. 2 strain (see table) showed that the latter was distinctly the more virulent. This capacity for producing haemorrhagic enteritis may be regarded practically as an attribute peculiar to the dysentery group—typical and atypical. The lesion differs from the punctiform haemorrhages sometimes met with in the intestinal wall in various types of septicaemia.

From their occurrence in large numbers in early cases of acute dysentery, in the absence of the classical types, and their characteristic effects in animal experiments (which were found to be similar to those produced with the classical Shiga and Flexner-Y types), together with their close biological similarity to organisms of the typical dysentery group, it was concluded that these atypical organisms were to be regarded as true dysentery bacilli<sup>1</sup>. This group, of course, includes organisms which have been designated "inagglutinable Shiga and Flexner types," but it would appear more rational to avoid this designation and class all the organisms with the group characters given above as atypical or paradysentery bacilli. In this group mutations and also late fermentations were frequently noted, and organisms which would on first examination have been designated inagglutinable Flexner varieties soon developed additional fermentative characters.

#### CLINICAL SIGNIFICANCE OF ATYPICAL *B. DYSENTERIAE*.

As regards the type of infection due to these organisms, the majority of the cases were of the milder type and the proportion of cases with the typical acute signs, *i.e.* passing of blood and mucus, was lower. Nevertheless severe types of dysentery were not infrequently met with apparently due to these varieties. To sum up, the Shiga infections were mostly of the severe type and the atypical *B. dysenteriae* infections of the milder type, while the Flexner-Y infections occupied an intermediate position in this respect. Atypical organisms have been isolated from stools within a few minutes from the time they were passed, and were the predominating organism present. Thus there is no support for the suggestion that these are accidental contaminations in faeces kept for some time.

<sup>1</sup> In the case of infections with *B. Shiga* and *B. Flexner* the agglutination reaction of the patient's serum was found to be so variable and unreliable and so frequently absent, except in such low titres (1:50 or less) as to introduce the fallacy of a normal serum effect, that no investigation of this reaction was carried out in the case of the atypical infections.

## MIXED INFECTIONS.

In certain instances mixed infections with typical and atypical organisms were noted, but these were relatively uncommon.

METHOD OF ISOLATING *B. DYSENTERIAE*.

It is not out of place here to refer briefly to the isolation of the dysentery bacilli. The medium I have generally employed and found most satisfactory has been MacConkey's agar. The use of a modified MacConkey's medium containing trypsinised heart extract did not appear to give appreciably better results. On MacConkey's medium the colonies of the Shiga bacillus were usually the smallest, and those of the atypical organisms the largest, but considerable variations were noted. The faeces must be examined as soon as possible after evacuation; repeated cultures from numerous specimens have shown that in general at room-temperature (Egypt in winter) the dysentery organisms cease to be recoverable after six to eight hours, although exceptionally they may persist much longer.

## FREQUENCY OF DYSENTERY BACILLI IN THE FAECES AT DIFFERENT STAGES OF THE DISEASE.

During the first few days of the illness, the dysentery bacilli were present in enormous numbers and often in almost pure culture; after this they tended to disappear and to be replaced by "concomitant organisms," see Table II, *e.g.* *B. Morgan* No. 1 and other similar organisms (B.C.L.A., Nos. 1, 2, 3, etc.),

Table II.

*Concomitant Bacilli.*

					Motility	Glucose	Lactose	Dulcité	Saccharose	Mannite	Maltose	Indol	Gelatin
<i>B. Morgan</i> No. 1	...	...	...	+	+	-	-	-	-	-	-	+	-
B.C.L.A. No. 1	...	...	...	+	+	-	-	-	-	-	-	-	-
„ No. 2	...	...	...	-	+	-	-	-	-	-	+	+	-
„ No. 3	...	...	...	-	+	-	-	-	-	-	-	+	-
„ No. 6	...	...	...	+	+	-	-	-	-	-	+	+	-
„ No. 7	...	...	...	-	+	-	-	-	-	-	-	-	-
<i>B. faecalis alkaligenes</i>	...	...	...	+	-	-	-	-	-	-	-	-	-
<i>B. paracolon</i> types	...	...	...	+	+	-	+	-	-	+	+	+	-
<i>B. proteus</i> types	...	...	...	+	+	-	-	+	-	-	+	+	+

All gram-negative bacilli.

The cocco-bacillus referred to in the text is morphologically coccial, but with only a few bacillary forms.

+ = acid and gas in the fermentation tests.



*B. faecalis alkaligenes*, *B. paracolon* types, *B. proteus*, *B. pyocyaneus*, *Staphylococci*, and a Gram-negative non-motile non-carbohydrate-fermenting coccobacillus. Thus at a later stage of a dysenteric illness, plate cultures showed large numbers of colonies of these concomitant bacilli and dysentery bacilli were absent. It is, of course, difficult to determine the actual part played by these organisms, but it would appear as if the dysentery bacilli proper often only initiated the lesions and that these other organisms acted in aggravating or maintaining the disease. In a considerable number of autopsies at various stages in which cultures were made directly from the floor of intestinal ulcers and from necrotic tissue no dysentery bacilli were isolated, but cultures of these various concomitants were obtained. These results render the significance of bacteriological findings in convalescent cases of extremely dubious value so far as throwing light on the etiology of bacillary dysentery is concerned.

## THE ASSOCIATION OF *RICKETTSIA* WITH TRENCH FEVER.

BY J. A. ARKWRIGHT, M.D., A. BACOT AND F. MARTIN DUNCAN.

(*From the Lister Institute of Preventive Medicine.*)

(With Plates II and III.)

THE following contains a preliminary account of the work on the Etiology and Pathology of Trench Fever which has been done at the Lister Institute in connection with the War Office Committee on Trench Fever under the Chairmanship of Major-General Sir David Bruce, F.R.S. A first account from the Clinical side has been published in a paper by Major Byam, R.A.M.C., and his colleagues at the New End Military Hospital, Hampstead, and read before the Society for Tropical Medicine and Hygiene in May of last year. We have been mainly dependent for our material on Major Byam and the rest of the Medical Staff of the New End Hospital and are very much indebted to them for their help and courtesy. We are under an especial obligation to Lieut. Ll. Lloyd, R.A.M.C., who has carried out that part of the entomological work done at the Hospital, taking immense pains in supervision and in obtaining the material which we required.

### HISTORY AND DESCRIPTION OF *RICKETTSIA* BODIES.

The class of microorganism to which the name of *Rickettsia* has been given by da Rocha-Lima is associated chiefly with two human diseases, Typhus and Trench Fever and the lice which transmit them. The parasites found in Rocky Mountain Spotted Fever are probably very closely related; they are described as occurring in blood films and in very large numbers in the Tick, which transmits this disease, by Ricketts (1909), Wolbach (1916, 1918). A fourth species has been found by Nöller (1917) in the "sheep tick," *Melophagus ovinus*; this species, *R. melophagi*, is not known to be associated with any mammalian disease. Töpfer (i. 1917) further associates war nephritis with another form of *Rickettsia*.

The chief characters distinctive of *Rickettsia* are the following: (1) morphology: they are of very small size,  $0.3$  to  $0.5 \times 1.5$  to  $2.0$  microns. Their shape resembles a coccus, diplococcus or a short bacillus. They stain rather feebly by aniline dyes, do not retain Gram's stain, and are not acid fast, but stain well by Giemsa, when they appear as small dots, double cocci, or bipolar staining bacilli with an unstained central part. They are non-motile. (2)

Occurrence in the blood: they occur in blood films sparsely, and are best seen in dehaemoglobinised thick drops taken during the periods of fever (Rocky Mountain Fever, Ricketts, 1909; Typhus, Ricketts and Wilder, 1910; Trench Fever, Töpfer, x. 1916). It is, however, now generally acknowledged that the recognition of scanty *Rickettsia* in the blood or tissues is very difficult and uncertain. (3) Occurrence in the insect vector: Ricketts and Wilder (1910) found these microorganisms in the louse in Mexican Typhus. Sargent, Foley and Viallatti (1914), da Rocha-Lima (1916), Töpfer and others confirmed this observation and described enormous numbers of the parasite in the midgut of lice that had bitten a patient during the height of the fever a few days previously. (4) Artificial culture: attempts to cultivate *Rickettsia* on artificial media have been usually unsuccessful, but Nöller records that he grew *R. melophagi* on a blood agar medium. The claims made by Töpfer (1917) and Csernel (1916) appear to have been based only on single occurrences and are unconfirmed.

There has been a tendency to regard *Rickettsia* as a Protozoon as first suggested by Prowazek and favoured by da Rocha-Lima. The reasons for this view appear to be largely *a priori*, on account of the fact that it is insect-borne, the relapsing character of the fever in Trench Fever, and in addition the peculiar staining properties attributed to the bodies by da Rocha-Lima. However Ricketts, Wilder, Töpfer and the present writers find that with Giemsa the staining reaction is very like that of other bacteria.

Nevertheless, this class of microorganism and its associated diseases appear to have sufficiently distinct characteristics to justify the retention of the name *Rickettsia* for the present.

#### THE ASSOCIATION OF TRENCH FEVER AND *RICKETTSIA* BODIES.

The presence of the virus of Trench Fever in the blood of patients during and just after a febrile attack was shown by McNee, Renshaw and Brunt (12. ii. 1916) by transmitting the disease to man by intravenous or intramuscular injection of blood. This has been confirmed and amplified by the War Office Committee on Trench Fever in England, and the American Red Cross Medical Research Committee in France (1918); the latter also made additional experiments on plasma, filtered material, etc. A few inoculation experiments in Germany (Werner, Benzler and Wiese (ix. 1916)) have also been published. Several observers (Jungmann (iii. 1916), Töpfer (iii. 1916) and others) claim to have seen definite bodies in the blood in wet and dry preparations which resemble diplococci or bipolar staining bacilli and are like those described by Ricketts in Rocky Mountain Spotted Fever, and by Ricketts and Wilder in Typhus.

The suspicion that lice were concerned in the spread of Trench Fever was suggested in McNee's paper, and has been supported by the evidence of Davies and Weldon (3. ii. 1917). Jungmann and Kuczinski (iii. 1917), Werner and Benzler (v. 1917) also make statements on the subject, which, however, do not afford very satisfactory proof of this mode of transmission.



The War Office Committee working in England (Byam (v. 1918)) and the American Medical Research Committee in France have shown conclusively that the disease is readily transmitted by lice from patients to healthy volunteers. The former Committee has shown that the excreta or the body contents of infected lice, rubbed into a scarified area on the skin of a healthy man almost invariably reproduces the disease, usually with relapses, and other characteristic symptoms after an incubation in man of six to 14 days. They also showed that infected lice fed for over 30 days on several healthy men failed to infect them, although the lice were frequently fed on Trench Fever patients during that time. The latter Committee found that when infected lice were allowed to feed on healthy men the majority contracted Trench Fever after a period of about 2-4 weeks had elapsed.

Töpfer (17. x. 1916) described microorganisms in lice from Trench Fever patients which very closely resembled those found in lice from Typhus Fever, but claimed that he could distinguish the two kinds of parasites—those associated with Trench Fever being shorter and thicker than those from Typhus lice, but he did not consider important da Rocha-Lima's distinction between Typhus and other kinds of *Rickettsia*, namely, the invasion of the epithelial cells of the gut wall by the former. He states that some of the lice caught on every Trench Fever patient contained these parasites, and that uninfected lice fed on Trench Fever patients were found subsequently to harbour similar forms. These microorganisms were first found in the midgut of lice on the 5th day after the first infecting feed and were present in very large numbers on and after the 8th day. Not every louse was found to be infected.

Jungmann and Kuczinski (23. iii. 1917) state that they regularly produced a fatal infection in wild mice with the blood of Trench Fever patients injected intraperitoneally and that they found the same bipolar staining parasites in the peripheral blood of the mouse and in the patients' blood. They also, like Töpfer, found that the guts of lice, which had fed on patients, became infected with parasites, but they could not distinguish them from those found in Typhus lice. They say that in both diseases the microorganism develops inside the epithelial cells of the louse's midgut. They state that the gut of an infected louse injected intraperitoneally into a mouse causes its death in the same way as when a patient's blood is inoculated. They infected normal lice by letting them bite Trench Fever patients, and both these and also 80 % of the lice caught on Trench Fever patients were found to be infected on microscopical examination.

Munk and da Rocha-Lima (30. x. 1917) confirm the occurrence and multiplication of *Rickettsia* in lice fed on Trench Fever patients. Da Rocha-Lima claims that parasites found in lice fed on patients suffering from the two diseases can be distinguished by the position in and destruction of the insects' gut-cells by *Rickettsia prowazeki* which is associated with Typhus, and the irregular and only occasional entrance into the cells by *Rickettsia*

*quintana* and *R. pediculi* which occur in lice from Trench Fever cases and normal lice respectively. He acknowledges that he is unable to distinguish *R. quintana* from *R. pediculi*. He maintains that the inability of other workers to distinguish *R. prowazeki* from the other two species is due to their examination having been made by means of smears, whereas a differentiation can only be made with certainty by examining serial sections 3-5 microns thick, of which he claims to have examined over 25,000. He also describes slight morphological differences.

#### ANIMAL EXPERIMENTS.

Da Rocha-Lima states that he infected guinea-pigs and produced a characteristic temperature chart by inoculating various kinds of material (blood, urine, lice) from patients. However, only seven out of 44 animals reacted typically, ten slightly and 27 not at all. He was unable to pass the disease on to other guinea-pigs from those first infected. He could not infect mice. He records 119 experiments made by feeding normal lice on 103 persons suffering from Trench Fever, or other fevers, or who were normal. He used lice from places which were believed to be free from Typhus, or lice bred in captivity. Lice which showed doubtful appearances or only scanty *Rickettsia* were excluded from the results. They were examined partly by smears and partly by serial sections. Of 70 experiments on Trench Fever patients 51 gave a positive result, *i.e.* at least one louse was found to contain *Rickettsia*. In 11 the lice remained negative. In 33 control experiments on men not suffering from Trench Fever, but who were in hospital with other diseases or were healthy, the lice fed on 26 gave negative results and those from six positive results; the lice from one of these latter were heavily infected though the patient had never had any general symptoms.

Of 14 persons examined in Hamburg, where there had been no Typhus fever, in two the majority of the lice were strongly infected. One of these was a man who had been associated with soldiers from the front.

Trench Fever patients were able to infect lice with *Rickettsia* both before, during and after a febrile attack, and after many weeks of convalescence.

#### TIME OF APPEARANCE OF *RICKETTSIA* IN THE LOUSE.

After the first experimental infecting feed on a patient, *R. quintana* was commonly found in lice on the 3rd to the 6th day, sometimes in large numbers on the 4th day. Lice caught on patients showed the parasites usually on the 6th to the 8th day. *R. prowazeki* was commonly well seen in lice after the 8th day from the first experimental feed on Typhus patients, and only exceptionally as early as the 4th or 5th day.

Trench Fever lice remained healthy, but Typhus lice often died. Lice infected with *R. quintana* remained infected after long continued feeding on a healthy man.

Da Rocha-Lima failed to infect by the bites of lice containing many



*Rickettsia* of the *R. pediculi* type. Korbseh (1916) reports a failure after two "infected" lice had fed on him for eight days, and also no result following scarification and rubbing in of a single "infected" louse on two occasions.

Strisower (iv. 1918) described three cases of transmission of Trench Fever to men by feeding infected lice on them and also claims to have infected mice and cats in series. Several other writers (e.g. His and Stintzing) criticise the work on *Rickettsia* adversely and support the hypothesis of a spirochaete as the infective agent.

#### DISCUSSION OF THE FURTHER EVIDENCE IN THE LITERATURE AS TO THE CAUSAL RELATION BETWEEN TRENCH FEVER AND *RICKETTSIA*.

(1) The hypothesis that *Rickettsia* causes Trench Fever is largely founded on the analogy of Typhus Fever in which the evidence brought forward is more complete owing to experiments on monkeys and guinea-pigs. The claims to have infected guinea-pigs, cats, mice and rabbits with Trench Fever in a recognisable form are very unconvincing in the published reports.

(2) Observations on forms resembling *Rickettsia* in the blood of Trench Fever patients are very difficult to interpret and unsatisfactory, since these forms are so scanty and their morphology alone when present in small numbers in a film is inconclusive.

(3) The inability to obtain growth of *Rickettsia* on artificial culture media make inoculation and re-infection experiments, after several subcultures, impossible at present.

(4) The chief reasons for the failure to obtain acceptance or a better hearing for *Rickettsia* as the probable cause of Typhus and Trench Fever are: (a) The not very rare occurrence according to Continental observers of *Rickettsia* in lice from sources where Typhus and Trench Fever have not been suspected; (b) connected with this objection, and perhaps largely the cause of it, is the difficulty in distinguishing the forms of *Rickettsia* associated with Typhus and Trench Fever from each other, and from those found in "normal" lice. There is no agreement as to definite means of distinguishing these parasites, but da Rocha-Lima's claim to differentiate them by their position in serial sections is more convincing than Töpfer's very slight description of differences in morphology. (c) The confusion has perhaps been enhanced by the failure of the German observers to work with a clean stock of lice. Da Rocha-Lima did use some lice bred in the laboratory, but he makes no statement as to their number or the proportion of such lice used or the results obtained with them, as distinguished from lice caught in surroundings believed to be free from infection. Moreover, the captive lice would require feeding on some human being and the infection of laboratory workers with Trench Fever is common.

The wide distribution and high incidence of Trench Fever on the Continent during the War makes it very difficult to obtain definitely uninfected lice for experiments or uninfected men on whom to feed them, except by breeding



lice, feeding them on a man of unimpeachable freedom from Trench Fever infection, and watching their excreta through several generations.

(5) The counter-claims of other workers to have discovered the cause of Trench Fever in a spirochaete mainly rest on the very insecure basis of occasional observations of a single spirochaete in the blood, supported by *a priori* arguments, except in the case of (1) Riemer (i. 1917) who, in addition, obtained these organisms in culture from one patient, but his observations have been unconfirmed, and (2) Couvy, Dujarric and de la Rivière (12. i. 1918), who experimentally infected guinea-pigs with patients' blood and passed the infection on to other guinea-pigs; these observers recovered the spirochaetes in considerable numbers from the kidneys, etc. Their observations have so far not been corroborated by other workers.

#### THE AUTHORS' OBSERVATIONS AND EXPERIMENTS.

Early in 1918 it had become clear from the experimental work on volunteers of the Committee (Byam 1918) that the virus of Trench Fever was contained in a very active form in the excreta and bodies of lice, since the body contents of 11 lice or a small pinch of dry excreta was sufficient to infect a man through the excoriated skin.

Much microscopic and cultural work had been done on the blood of patients by previous workers, at the New End Hospital, Hampstead, especially by Captain Dimond, and, since its formation, by the members of the War Office Committee.

It was determined therefore early in May, 1918, to concentrate our attention on the excreta and contents of the intestinal canal of infected lice, while some cultural and other observations on the blood of patients were continued.

Cultures of the excreta and guts of lice, both infected and normal, had sometimes yielded a variety of bacteria and sometimes been sterile, but one form of *Bacillus* was the most constant and had been previously studied by Bacot. In young cultures on agar or blood agar it takes the form of a coccus 1.5 to 2.0 microns in diameter and is often seen dividing. In older cultures, and sometimes even in the first 24 hours, films show irregular threads amongst the round or oval organisms. When first isolated growth takes place best at about 27° C., but later it grows well at 37° C. It is Gram-negative and non-motile; ferments glucose, mannite and lactose very slowly; forms acid and clot in milk in about 14 days, and is not pathogenic for guinea-pigs. Attempts were then made by means of wet and dry films and by cultures to discover other organisms in the excreta of infected lice which were not present in those of normal lice.

One or two films made by the late Professor Plimmer from the excreta of infected lice and fixed wet with formalin and iodine vapour showed an immense number of very small particles which were probably minute organisms, and turned our attention to a search for the *Rickettsia* described by Töpfer and Rocha-Lima. In many films of excreta, whether fixed wet or dry, a large

number of stained granules were seen, which recalled descriptions of *Rickettsia*, but it was very difficult to distinguish the granules of altered blood, etc., from the more definite forms, and one was continually in doubt as to whether one was looking at minute microorganisms or precipitated protein. In order to get rid of the débris of red corpuscles, the films after drying were fixed for one or more hours in absolute alcohol containing 20 drops of strong hydrochloric acid per c.c. as recommended for thick blood drops in Malaria work by W. M. James. By using this technique, there has seldom been difficulty in distinguishing *Rickettsia* when present from granules of débris, though the disintegrating nuclei of leucocytes occasionally have presented a somewhat similar appearance. The chief further source of error lies in the danger of confusing these parasites with other microorganisms, especially larger bacteria only stained in the centre or at the poles, and with the small bacteria occasionally seen, which approach *Rickettsia* in size.

The criteria which we have adopted in deciding on the presence of *Rickettsia* have been (1) its minute size, smaller than *M. melitensis* or *B. influenzae*, usually about  $0.3 \times 0.3$ , or  $0.3 \times 0.5\mu$ ; (2) its irregularity in shape, round, oval, diplococcal or bacillary with stained poles; (3) its occurrence in very large numbers, or even in masses, especially on flakes of solid material in the excreta; (4) its well-stained appearance when coloured by Giemsa, the colour being purple like that of the nucleus of a leucocyte.

When small granules, somewhat resembling *Rickettsia* in size and shape, but stained pink or lilac, have been met with, or if the bodies have been very few and scattered about the film, the result of microscopical examination has been recorded as negative or doubtful. The number of "doubtful" specimens has very much diminished as our experience has increased, and now it is very seldom necessary to return an uncertain result, though *Rickettsia* no doubt are sometimes present but unrecognized on account of their rarity in the film.

#### METHOD OF CONDUCTING EXPERIMENTS WITH LICE.

A large, healthy stock of *Pediculus corporis*, maintained by Bacot for over three years, was used. The lice were confined in boxes, covered with fine gauze, and were fed twice daily by the method described by him (Bacot, 1917). When not in use the lice were kept in an incubator or in an inside pocket at  $27^{\circ}$  to  $30^{\circ}$  C. except in those experiments in which another temperature is recorded. The lice in each box usually numbered 50 to 100. They were fed once or twice for 20 to 30 minutes on Trench Fever patients during an attack of fever, also in some instances during non-febrile periods; thereafter they were fed on a healthy man or in some experiments on the same infected man throughout.

Excreta were examined by shaking them as a dry powder out of the boxes through the gauze, and making an emulsion on a glass slide with a drop of salt solution. The contents of the gut were examined by dissecting it out



and emulsifying it on a slide with needles in a small drop of salt solution. The film was dried and fixed in acid alcohol and stained with Giemsa. Dead lice were also examined by soaking the body in a drop of salt solution and teasing it up on a slide. It was found that films made from dead lice showed *Rickettsia* very clearly when these were present.

Some infected and uninfected lice were also examined by serial sections.

#### INCUBATION OF *RICKETTSIA* IN THE LOUSE.

It was found that if a boxful of lice were given an infecting feed the excreta obtained from the box did not show *Rickettsia* for some days.

Table I.

Showing the results of examining microscopically the excreta of lice on a series of days after the first infecting feed, and also the results of inoculation of excreta from two of the boxes, 150 and 134.

Days from first infecting feed	Box No.								150		134	
	175	142	A 15	A 18	A 25	A 34	193	A 33*	Microsc. exam.	Result of inoculation	Microsc. exam.	Result of inoculation
1st	—	—	...	—	—	—	...	—	—	—	—	—
2nd	...	+ ?	...	—	—	—	...	—	...	...	...	...
3rd	...	—	...	—	—	—	—	—	...	...	— ?	—
4th	+	—	...	...	—	—	—	—	...	...	...	...
5th	+ ?	—	—	—	—	...	—	...	+	+	— ?	—
6th	—	—	...	+ ?	—	—	—	—	—	—	...	...
7th	...	+	...	—	—	—	—	—	++	+	...	...
8th	...	+	+++	+++	—	—	—	—	—	—	+	—
9th	+	+	++	...	—	+	—	—	+	+	...	...
10th	++	+	...	+++	+++	+	—	—	+	+	...	...
11th	...	+	+++	+++	+++	+	+	—	+	+	...	...
12th	++	+	...	+++	+++	+	+	—	...	...	...	+
13th	...	+++	+++	+++	+++	+++	++	—	...	...	+	...
14th	...	+++	...	+++	...	++	+++	...	...	...	...	...
15th	...	...	...	+++	...	++	++	—	...	...	...	...
16th	++	+++	+++	+++	...	...	+++	—	...	...	...	...
17th	...	++	...	+++	+++	+++	++	—	...	...	...	...
18th	...	++	...	+++	...	...	...	...	...	...	...	...
19th	...	+	...	+++	+++	...	...	...	...	...	...	...
20th	+++	+	...	+++	+++	...	+++	...	...	...	...	...
21st	...	...	...	+++	+++	...	...	...	...	...	...	...
22nd	...	...	...	+++	...	...	...	...	...	...	...	...
23rd	...	...	...	...	...	...	...	...	...	...	...	+
24th	...	...	...	...	...	...	...	...	...	...	+	...

+ = *Rickettsia* seen in a few microscope fields.

++ = " " several " "

+++ = " " enormous numbers.

— = No *Rickettsia* seen.

... = No examination.

\* The lice in boxes A 33 and A 34 were fed at the same time, but A 33 was kept at about 20° and A 34 at 27° C.



When *Rickettsia* have once been found in a box in large numbers they usually continue to be found in daily examinations till all the lice of the infected generation are dead.

Table I shows the results of a daily microscopical examination of the excreta from ten of the boxes of experimental lice which had previously fed on a Trench Fever patient. The results of a series of experimental inoculations into volunteers of the excreta from two of the same boxes, 150 and 134, are also shown. It is seen, (1) that *Rickettsia* appear in the excreta after a series of negative examinations following the infecting feed, and (2) that the numbers of these bodies can often be seen to be smaller when they first appear than on later days, (3) also that when the infection is thoroughly established a positive result is obtained every day. The period elapsing between the first infecting feed and the recognition of *Rickettsia* varies from about 4 to 10 days in this series. Examinations of boxes kept at about 20° C. remained consistently negative; though the lice were apparently healthy.

The results shown in this table, *i.e.* an incubation period, the appearance of *Rickettsia* after about a week or ten days, and its persistence for three or four weeks, have been a constant phenomenon when we have been able to examine a series of specimens.

Table II relates to experiments with 20 boxes of lice which were fed on ten different patients with Trench Fever. The boxes were examined at frequent intervals, in most cases daily, either by making films from the excreta, or from lice. It is seen that *Rickettsia* was first seen on the 5th to the 12th day, most commonly on the 7th to the 10th day from the first infecting feed, when the lice were kept at about 27° C. between the feeds. Boxes A 20 b, A 28, A 30, and A 33 were kept at about 20° C. and *Rickettsia* did not appear, though examinations were continued till the 17th, 19th and 22nd day from the infecting feed in these experiments. It was also shown that if a box was kept at 20° C. for two or three days and then at 27° C. the parasites were found after a week at the higher temperature.

Lice in eight other boxes fed on seven infected patients (six of whom were different men from those mentioned in Table II) have been found to be infected with *Rickettsia* after a variable number of days, but the examinations have not been sufficiently numerous to determine the date when these first appeared. In two of them, however, the parasites were present in the excreta on the 6th day and in one on the 7th day from the first infecting feed.

Seven other boxes of lice, which have been fed on patients believed to be suffering from Trench Fever, have only been examined on two or three occasions, and *Rickettsia* has not been definitely found.

Table II also shows the number of days which elapsed after an infecting feed before the excreta were proved to be infective, *i.e.* capable of producing Trench Fever when inoculated into volunteers in two series of experiments.

In the case of Box 134 the excreta from the 1st to the 8th day did not infect, but those collected on the 12th day reproduced the disease, whereas

*Rickettsia* were first demonstrated on the 8th day. The excreta from Box 150 showed *Rickettsia* on the 5th day and the same specimen of excreta infected a volunteer. Excreta from Box A 18 showed the parasite microscopically on the 8th day, and a mixed sample of excreta collected on the 8th to the 22nd day proved virulent for man, but they were not tested earlier for virulence.

Table II.

Showing the day after the first infecting feed on which *Rickettsia* was first found in the guts or excreta of lice.

In the case of Boxes 134 and 150 the day when infective excreta were first obtained is also shown.

No. of boxes of lice	Source of infection of lice			Material examined	Day from infecting feed on which	
	Ref. No. of patient	Day of disease	Febrile or non-febrile		Microscopic result first positive	Successful inoculation obtained
162	{ Ex. 46 Ex. 21	2nd 11th	Febrile Non-febrile	Midgut	10th	...
175	Ex. 60	1st	Febrile	"	9th	...
134	G.	79th	"	Excreta	8th	12th*
150	Ex. 33	2nd	"	"	5th	5th*
142	Ex. 27	2nd	"	"	7th	...
A 15	A.	2nd	"	"	8th	...
A 16	A.	5th	Non-febrile	"	5th	...
A 18	A.	10th	Febrile	"	8th	...
A 20 a†	A.	23rd	Non-febrile	"	10th	...
A 24	Ex. 73	25th	Slightly febrile	"	9th	...
A 25	C.	43rd	Non-febrile	"	10th	...
A 27	A.	29th	"	"	12th	...
A 29‡	Ex. 81	19th	Febrile	"	9th	...
A 31§	Ex. 81	30th	Slightly febrile	"	7th	...
A 34	A.	49th	Non-febrile	"	9th	...
A 35	A.	49th	"	"	7th	...
A 20 b†	A.	23rd	"	"	Negative	19th
A 28‡	Ex. 81	19th	Febrile	"	"	17th
A 33	A.	49th	Non-febrile	"	"	17th
A 30§	Ex. 81	30th	Slightly febrile	"	"	22nd

\* See also Table I.

† Boxes A 20 a and A 20 b were alike and treated in the same way except that A 20 a was kept at 27° C. and A 20 b at 20° C.

‡ Boxes A 28 and A 29 were alike and treated in the same way except that A 28 was kept at 20° C. and A 29 at 30° C.

§ Boxes A 30 and A 31 were alike and treated in the same way except that A 30 was kept at 20° C. and A 31 at 27° C.

|| Boxes A 33, A 34, A 35 were alike and treated in the same way except that A 33 was kept at 20° C., A 34 at 27° C. and A 35 at 32° C.

These facts show a general agreement in that the virus of Trench Fever and *Rickettsia* both require an incubation period of 4 to 12 or more days in the louse before they are demonstrable—the former by inoculation, the latter microscopically. This point will be dealt with in more detail further on.

The lice in Boxes 162, 175, 134, 150 and 142 were fed on the infected man







In Series 1 comprising lice from six boxes the lice only fed on an infected man during 24 hours, and afterwards on a healthy man. In Series 2 (eight boxes) the lice fed on an infected man from the first infecting feed onwards. When individual lice from a recently infected box are dissected and examined it is found that only a few are infected with *Rickettsia* in the first week after the first infecting feed, that during the second week about an equal number are infected and uninfected, and that after the second week the majority show parasites microscopically.

Table III gives the actual figures in a series of lice taken at different stages from 14 boxes of infected lice, and examined microscopically. The increase in the number of infected lice is very marked.

In microscopic sections of infected lice *Rickettsia* were seen crowding the region of the epithelial cells lining the alimentary canal, but there was no definite invasion of the cells. Appearances like those figured by da Rocha-Lima from sections of Typhus lice in which the epithelial cells show well defined areas which are badly stained and occupied by masses of *Rickettsia* were not seen.

#### EXAMINATION OF NORMAL LICE.

For comparison with these boxes of lice which have been infected by feeding on Trench Fever patients a number of boxes of uninfected lice have also been examined frequently, some daily for weeks, by making films of the excreta. Also a considerable number of normal lice have been dissected and films from the midgut examined, and serial sections have been cut of others. Only in one box on one occasion have forms closely resembling *Rickettsia* been found. Seeing how difficult or impossible it is to distinguish bacteria by their morphology, an occasional error is not surprising. When these organisms occur in an infected box they are almost always found on many successive days and not only on one single occasion, as was the case with the apparently exceptional occurrence among normal lice mentioned above.

In all 22 boxes of lice fed on normal persons have been examined repeatedly over periods lasting usually over two months and never less than 14 days. These normal persons on whom they have been fed are seven in number, and two stocks of normal lice from different sources are under observation.

Two other boxes of lice supposed at the time to be normal, besides the one referred to above, showed *Rickettsia*. They were both being fed on A. who had been also feeding infected lice for over five weeks and had been working daily with infected excreta in the laboratory. He developed Trench Fever on June 11th. The first box, A 12 a, showed *Rickettsia* in the excreta on June 8th, and of nine lice dissected on June 10th three gave a positive result, the other box, A 10, showed infected excreta on June 14th. It does not seem reasonable to include these findings in Boxes A 10 and A 12 a as positive results from normal lice.

Some other lice found on healthy civilians have been examined but none have been found to be infected with *Rickettsia*.

The total number of specimens of lice or excreta from boxes of lice which had been fed on men believed to be infected with Trench Fever was 253, and of these 150 showed *Rickettsia*, 83 gave a negative and 20 a doubtful result. Of these specimens of excreta collected during the first week after the first infective feed, 14 were positive and 73 were negative.

Second week, 75 were positive and 27 were negative.

Third week, 61 were positive and 3 were negative.

Of 245 specimens from 22 boxes of normal lice fed on seven healthy persons, only one was positive (if the four specimens from A's two boxes mentioned above are excluded), 234 gave a negative and 10 a doubtful result.

#### CORRELATION OF THE PRESENCE OF *RICKETTSIA* AND TRENCH FEVER VIRUS IN THE LOUSE.

The association of *Rickettsia* and infectivity for man of the lice containing them is very striking; *Rickettsia* and the virus of Trench Fever also have certain properties in common.

1. The size of the *Rickettsia* is such that one would expect them to be held back by a good Berkefeld filter; they nevertheless approach the lower limits of size of known bacteria.

In an experiment by McNee with filtered and unfiltered blood plasma, the unfiltered alone transmitted the disease. The American Medical Research Committee (1918) in France have been able to transmit the disease by means of filtered material. They have also found that plasma, freed from cells by centrifuging, is still virulent. In experiments with emulsion of louse excreta in salt solution, we have found that only prolonged centrifuging at high speed (2500–3000 revolutions) for 20–30 minutes will produce a definite deposit of *Rickettsia*. They may therefore be separable from blood corpuscles by fractional centrifuging in blood plasma also, but we have been unable by centrifuging to demonstrate their presence in more than small numbers in citrated plasma of Trench Fever patients diluted 1 in 5.

2. The blood in Trench Fever is infective by direct inoculation during a febrile attack, and, at any rate sometimes, when there is no fever. Lice can also be infected with Trench Fever virus over a long period, whether the patient is febrile or not; they also become infected with *Rickettsia* when fed on patients during similar periods, *e.g.* the excreta of lice (Box 163) fed on the patient St. when non-febrile on the 27th to 28th day of the disease gave Trench Fever to several volunteers and contained large numbers of *Rickettsia*; the lice in Box 134 fed on another patient G. on the 79th day of his disease, gave Trench Fever to a volunteer by means of excreta collected on the 12th day from the infecting feed. The excreta from this box first showed *Rickettsia* on the 8th day.

3. Lice fed on patients are able to transmit the disease if their excreta or midgut contents are rubbed into scratches or inoculated subcutaneously. They, however, appear not to become infective (two series of experiments)



till the 5th to the 12th day after the infecting feed. In the same way *Rickettsia* is not in our experience recognizable in lice or their excreta when kept at 27° C. till the 5th to the 12th day (usually the 7th to the 10th day) after the first infecting feed on a patient.

4. In an infected box not every louse appears to contain the virus of Trench Fever as tested by inoculation of man, neither does every louse contain *Rickettsia* in recognizable form or amount. Only 12 out of 21 examined in the second week from the infecting feed showed these parasites. In one experiment two lice were selected from an infected box (A 16 a) on about the 13th day after the first infecting feed. The midguts were dissected out and emulsified separately. It was shown by microscopic examinations of both the gut contents and the excreta of these lice that the one, A, contained many *Rickettsia* and the other, B, did not. Inoculation of the emulsions separately into two volunteers by scarification, produced Trench Fever in the one inoculated with A and not in the one inoculated with B.

5. A box once infected appears to remain so for two or three weeks, both as regards the virus of Trench Fever and the presence of *Rickettsia*, i.e. during the life-time of the infected generation of lice.

6. The high infectivity of louse excreta is associated with the presence of enormous numbers of *Rickettsia*, whereas the difficulty of finding these bodies in blood films is well known.

7. Lice or the excreta of lice which have been proved to contain virus, as the result of successful inoculation, have in most cases, when examined, been shown to contain large numbers of *Rickettsia*. Certain exceptions to this have occurred. It is however not surprising that a small sample of excreta should occasionally fail to show the parasite which may be present in large numbers in another part of the same excreta. On the other hand, if uniformly distributed, *Rickettsia* might escape recognition though present in considerable numbers.

In Table IV are shown all the experiments made by inoculating volunteers with the excreta or body contents of lice in which the specimen has also been examined microscopically. The result of such examination is shown in column 3 and of the inoculation experiment in column 5. The 6th column states whether there is agreement (A.), disagreement (Dis.), a doubtful result (?) or a reason for agreement not being expected (O.).

Fifty-six specimens are included in Table IV. Fifty-three were samples from boxes of lice which had had a feed on an infected patient, of these 50 were samples of excreta and three were emulsions of single lice. Three further specimens of excreta are included in the Table—two from boxes of normal lice fed on healthy men and both examined microscopically and inoculated into volunteers, as control experiments; the third specimen is from a box containing the offspring of infected lice which were examined to test the hypothesis of hereditary transmission of the virus of Trench Fever or of *Rickettsia*. Out of the 53 specimens from boxes of lice which had been



Table IV.

Showing the results of microscopical examination and inoculation of 54 specimens of louse excreta and of three single lice.

1 No. of Box of Lice	2 Source of infecting feed		3 <i>Rickettsia</i> in specimen	4 Days from infecting feed	5 Inoculation of man			6 Agree- ment or dis- agree- ment	7 Incuba- tion in man. Day	Notes
	Name	Date			No. of Ex- peri- ment	Date	Result			
Mixed	...	...	+++	...	10	9. ii.	+	A.	6th	
Mixed	...	...	+++	...	11	9. ii.	+	A.	7th	
Mixed	...	...	+++	...	12	9. ii.	+	A.	9th	
Mixed	...	...	+++	...	20	5. iii.	+	A.	9th	
Mixed	...	...	+++	...	21	16. iii.	+	A.	8th	
134	G.	20. iii.	-	1st	23	21. iii.	-	A.	...	
134	G.	20. iii.	-	3rd	24	23. iii.	-	A.	...	
134	G.	20. iii.	-	4-5th	25	25. iii.	-	A.	...	
134	G.	20. iii.	+	* 6-8th	26†	28. iii.	-	Dis.	...	
134	G.	20. iii.	{ + }	12th 13th	27	1. iv.	{ + }	A.	8th	
Mixed	...	...	+++	...	28	28. iii.	-	O.	...	Immunity
Mixed	...	...	+++	...	29	30. iii.	-	O.	...	Immunity
134	G.	20. iii.	{ + }	23rd 24th	30	13. iv.	{ + }	A.	7th	
Mixed	...	...	+++	...	32	6. iv.	+	A.	8th	Exereta untreated
Mixed	...	...	+++	...	33	6. iv.	+	A.	8th	Exereta heated 56° C.‡
Mixed	...	...	+++	...	34	6. iv.	-	O.	...	Exereta heated 80° C.§
150	J.	15. iv.	-	1st	39	17. iv.	-	A.	...	
150	J.	15. iv.	-	6th	41	21. iv.	-	A.	...	
150	J.	15. iv.	++	7th	42	22. iv.	+	A.	13th	
150	J.	15. iv.	-	8th	43	23. iv.	-	A.	...	
150	J.	15. iv.	+	9th	45	26. iv.	+	A.	9th	
150	J.	15. iv.	+	10th	46	26. iv.	+	A.	7th	
Mixed	...	...	+	...	51	7. v.	+	A.	8th	
150	J.	15. iv.	+	5th	53	2. v.	+	A.	16th	Few <i>Rickettsia</i> ; none seen in 2nd film
150	J.	15. iv.	-	6th	{ 41 54 }	{ 2. v. 2. v. }	{ - - }	A.	...	
150	J.	15. iv.	++	7th	55	2. v.	+	A.	13th	
150	J.	15. iv.	+	9-10-11th	58	10. v.	+	A.	12th	
150	J.	15. iv.	+	11th	59	2. v.	+	A.	8th	
163	S.	7. v.	+++	11-12-13th	64	20. v.	+	A.	7th	
163	S.	7. v.	+++	21-22-23rd	70	1. vi.	+	A.	7th	
157	W.	23. iv.	+?	26-31st	71	31. v.	+	?	8th	One louse
155 } 164 }	T.	...	-	11-24th	72	1. vi.	+	Dis.	10th	
163	S.	7. v.	+++	11-14th	73	2. vi.	+	A.	9th	
163	S.	7. v.	+++	11-24th	74	5. vi.	-	O	...	Exereta treated with lysol 2 %

A.=results agree.                      Dis.=results disagree.                      O.=results not comparable.

\* A very few *Rickettsia* were found in only one out of three films.  
† This man was inoculated three times with negative result (Exps. 43 and 63).  
‡ Dry heat for 20 mins.  
§ Moist heat for 10 mins.

Table IV—*continued*.

Showing the results of microscopical examination and inoculation of 54 specimens of louse excreta and of three single lice.

1 No. of Box of Lice	2 Source of infecting feed		3 <i>Rickettsia</i> in specimen	4 Days from infecting feed	5 Inoculation of man			6 Agree- ment or dis- agree- ment	7 Incuba- tion in man. Day	Notes
	Name	Date			No. of Ex- peri- ment	Date	Result			
163	S.	7. v.	+++	11-24th	75	8. vi.	+	A.	10th	
170	C.	14. v.	+	12-20th	76	9. vi.	-	O.	...	Immunity
163	S.	7. v.	+++	11-13th	77	11-13. vi.	+	A.	11th	
163	S.	7. v.	+++	11-24th	80	14. vi.	+?	?	12th	Influenza (?)
163	S.	7. v.	+++	11-24th	81	14. vi.	+	A.	10th	
163	S.	7. v.	+++	14-36th	82	14. vi.	-	O.	...	Excreta heated to 70° C. 20 mins.—moist
171	B.	15. v.	+++	11-19th	85	16. vi.	-	O.	...	48th day of disease— immunity
177	P.	29. v.	-	12-20th	86	18. vi.	+	Dis.	9th	
173	T.	17-18-19. v.	±	14-17th	90	19. vi.	-	?	...	Immunity
150	J.	15. iv.	-	8th	91	19. vi.	-	A.	...	
155 } 164 }	T.	...	-	11-24th	92	22. vi.	-	A.	...	
173	T.	17-18-19. v.	±	14-17th	93	22. vi.	-	?	...	
163	S.	7. v.	+++	11-36th	94	2. vii.	-	O.	...	Excreta heated to 99·9° C. 20 mins.—dry
A 18	A.	20. vi.	+++	8-22nd	96	19. vii.	+	A.	7th	2 mg. excreta sub-cut.
A 18	A.	20. vi.	+++	8-22nd	97	19. vii.	+	A.	11th	0·1 mg. excreta sub-cut.
Mixed	R.	...	+++	...	98	23. vii.	-	O.	...	Immunity—excreta kept 154 days
171	B.	15-18. v.	+++	11-19th	99	23. vii.	+	A.	9th	
A 16 a	A.	13. vii.	-	11th	100	24. vii.	-	A.	...	One louse
A 16 a	A.	13. vii.	+++	11th	101	24. vii.	+	A.	9th	One louse
122	Healthy man		-	...	22	18. iii.	-	A.	...	Offspring of infected lice
127-9	Healthy man		-	...	17	12. iii.	-	A.	...	Lice were fed on a heal- thy man as a control experiment
127-9	Healthy man		-	...	18	12. iii.	-	A.	...	

A. = results agree.

Dis. = results disagree.

O. = results not comparable.

fed on Trench Fever patients, three gave uncertain results from microscopic examination and in one the reaction following inoculation was of a doubtful nature, as the attack of fever was attributed to influenza, nine are excluded from the results because agreement between the results of the two methods of examination was not to be expected, either on account of the disinfection of the excreta by lysol or heat, or because the volunteer had previously gone through an attack of Trench Fever and was probably immune—the inoculation being in fact given as a test for immunity.

Of the 40 remaining, 27 gave positive results, both as regards the presence of *Rickettsia* and the virulence of the samples, 10 gave negative results from both tests, and three different results from the two tests.

There was therefore agreement as to the presence or absence of *Rickettsia* and virus in 37 out of the 40 samples which gave a decided answer, *i.e.*

92.3 % of agreement. In one of the three samples in which there was disagreement the microscopic examination was positive, and the virulence test negative; in the other two the reverse was the case.

The specimens of excreta from which results showing agreement were obtained were in some instances inoculated into more than one man. There are eight such observations recorded which were repetitions of former experiments. In seven of these the two results were positive and in one negative. In every case the excreta when used for a second experiment gave the same result as on the first occasion. Excluding the repetitions the total number of experiments is 32 of which 29 showed agreement (90.6 %) and three disagreement.

8. There is evidence that the virus of Trench Fever is not inherited in the louse, since excreta of the offspring of infected lice fed on healthy men from the egg have failed on inoculation to produce Trench Fever; the excreta of the offspring of the infected lice in several boxes have on examination not shown *Rickettsia*. One apparent exception to this statement occurred (see above).

#### CONCLUSIONS.

1. The intimate association in lice of *Rickettsia* with the virus of Trench Fever appears to have been amply proved.

2. The examinations of lice which have fed on healthy civilians in England have given negative results in a sufficiently uniform manner to constitute a significant negative control, but further examinations of lice from normal civilians are desirable.

3. Lice from soldiers who have been in France, or who have mixed with men from France in this country, would not afford a satisfactory control, since the infection of Trench Fever with the power of infecting lice with Trench Fever virus and with *Rickettsia* may be very long lasting.

4. Whether *Rickettsia* constitute the virus of Trench Fever or are in some way produced by it remains undecided because *Rickettsia* cannot be cultivated on artificial media.

5. It is conceivable that *Rickettsia* are not living microorganisms, but their appearance certainly suggests that they are bacteria, and their remarkable association with Trench Fever virus in the louse further suggests that they are the causal agent of Trench Fever.

#### REFERENCES.

- American Red Cross Medical Research Committee. Report of Commission on Trench Fever* (1918). Oxford University Press.
- BACOT, A. (26. xi. 1917). A contribution to the bionomics of *Pediculus humanus (vertimenti)* and *Pediculus capitis*. *Parasitology*, IX. p. 228.
- BYAM, W. and others (v. 1918). Trench Fever, a Louse-borne Disease. *Trans. Soc. of Trop. Med. and Hygiene*, May 1918, XI. No. 7.
- COUVY, L., DUJARRIC, R. and DE LA RIVIÈRE (12. i. 1918). Note sur l'étiologie de la fièvre des Tranchées. *C. rend. Soc. Biol.* LXXXI. p. 22.



- CERNEL (28. xii. 1916). Ueber die Morphologie des Fleckfiebererregers. *Wien. klin. Wochenschr.* No. 52, p. 1643.
- DAVIES, F. C. and WELDON, R. P. (3. ii. 1917). A preliminary contribution on "P.U.O." (Trench Fever). *Lancet*, i. p. 183.
- HIS, W. (3. vii. 1916). Ueber eine neue periodische Fieber-erkrankung (Febris Wolhynica). *Berlin. klin. Wochenschr.* p. 738.
- JUNGSMANN, P. (iii. 1916). Zur Aetiologie des Febris Wolhynica. *Berlin. klin. Wochenschr.* p. 323.
- JUNGSMANN, P. and KUCZINSKI, M. H. (22. iii. 1917). Zur Klinik und Aetiologie der Febris Wolhynica (His-Wernersche Krankheit). *Deutsche med. Wochenschr.* p. 359.
- KORBSCH, R. (5. x. 1916). Zur Kenntnis der Febris Wolhynica. *Deutsche med. Wochenschr.* p. 1217.
- MCNEE, J. W., RENSCHAW, A. and BRUNT, E. H. (12. ii. 1916). "Trench Fever," A Relapsing Fever occurring with the British Forces in France. *Brit. Med. Journ.* p. 225.
- MUNK, F. and DA ROCHA-LIMA, H. (30. x. 1917). Klinik und Aetiologie des sogen. Wolhynischen Fiebers (Werner-Hissche Krankheit). II. Ergebnis der ätiologischen Untersuchungen und deren Beziehungen zur Fleckfieberforschung. *München. med. Wochenschr.* p. 1422.
- NÖLLER, W. (10. vii. 1916). Beitrag zur Flecktyphusübertragung durch Läuse. *Berlin. klin. Wochenschr.* p. 778.
- RICKETTS, H. T. (30. i. 1909). A Microorganism which apparently has a specific relationship to Rocky Mountain Spotted Fever. *Journ. Amer. Med. Assoc.* LII. p. 379.
- RICKETTS, H. T. and WILDER, R. M. (23. iv. 1910). The etiology of the Typhus Fever (Tabardillo) of Mexico City. *Journ. Amer. Med. Assoc.* LIV. p. 1373.
- RIEMER (16. i. 1917). Beiträge zur Frage des Erregers des Fünftagesfiebers. *München. med. Wochenschr.* LXIV. p. 92.
- DA ROCHA-LIMA, H. (22. v. 1916). Zur Aetiologie des Fleckfiebers. *Berlin. klin. Wochenschr.* LIII. p. 567.
- SERGEANT, EDM., FOLEY, H. and VIALLATTE, C. (13. vi. 1914). Sur des formes microbiennes abondantes dans le corps de poux infectés par le typhus exanthématique et toujours absentes dans les poux témoins, non typhiques. *Compt. rend. Soc. Biol.* LXXVII. p. 101.
- STINTZING, R. (30. i. 1917). Ueber Febris quintana. *München. med. Wochenschr.* p. 155.
- STRISOWER, R. (30. iv. 1918). Experimentelle u. klinische Beiträge zur Febris quintana. *Ibid.* LXV. p. 476.
- TÖPFER, H. (20. iii. 1916). Zur Aetiologie des Febris Wolhynica. *Berlin. klin. Wochenschr.* p. 323.
- (12. x. 1916). Der Fleckfieber-erreger in der Laus. *Deutsche med. Wochenschr.* XLII. No. 41, p. 1251.
- (17. x. 1916). Zur Ursache und Uebertragung des wolhynischen Fiebers. *München. med. Wochenschr.* No. 42, p. 1495.
- (24. vi. 1917). Ursache und Uebertragung der Kriegsnephritis. *Medizin. Klinik*, p. 678.
- WERNER, H., BENZLER, J., and WIESE, O. (19. ix. 1916). Zur Aetiologie des Fünftagesfiebers. *München. med. Wochenschr.* p. 1369.
- WERNER, H., and BENZLER, J. (22. v. 1917). Zur Aetiologie und Klinik der Febris quintana. *Ibid.* p. 695.
- WOLBACH, S. B. (1916-17). The etiology of Rocky Mountain Spotted Fever. *Journ. Med. Research*, XXXIV. No. 1, p. 121, XXXV. No. 1, p. 147.

## DESCRIPTION OF PLATES II AND III.

Figs. 1 and 3. Films of excreta of infected lice from Box A 16, made 30. vi. 18 on the 15th day from the first infecting feed on a Trench Fever patient.

Fig. 2. Film of excreta of infected lice from Box A 18, made 30. vi. 18 on the 10th day from the first infecting feed. Stained Giemsa.  $\times 1000$ . Showing pure "culture" of *Rickettsia*.

Figs. 4 and 5. Films made from the fore-gut of an infected louse from Box 171, made 10. vi. 18 on the 26th day from the first infecting feed. Showing numerous *Rickettsia*—pure.

Fig. 6. Film of excreta of normal lice fed on healthy man; showing bacteria which are sometimes found in the excreta of normal lice. Stained Giemsa.  $\times 1000$ .

Figs. 7 and 8. Sections of the hind-gut of an infected louse from Box 160, killed 4. v. 18, the 7th day after the first infecting feed. Stained Giemsa.  $\times 1000$ . Showing *Rickettsia* on the surface of the epithelium. *a*, body-cavity. *b*, gut-wall. *c*, lumen of gut containing altered blood, and, close to the epithelial cells, *Rickettsia*.

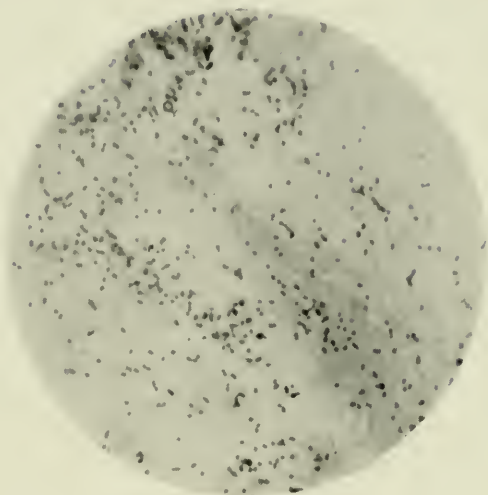


Fig. 1

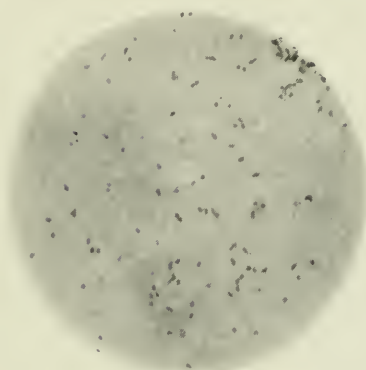


Fig. 2

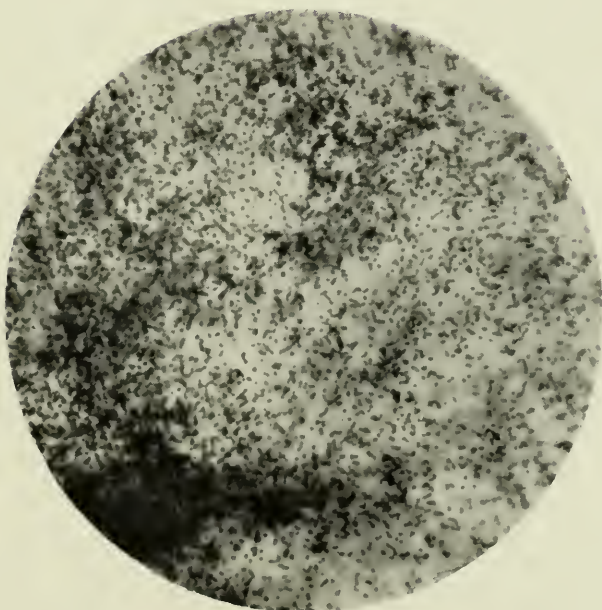


Fig. 3





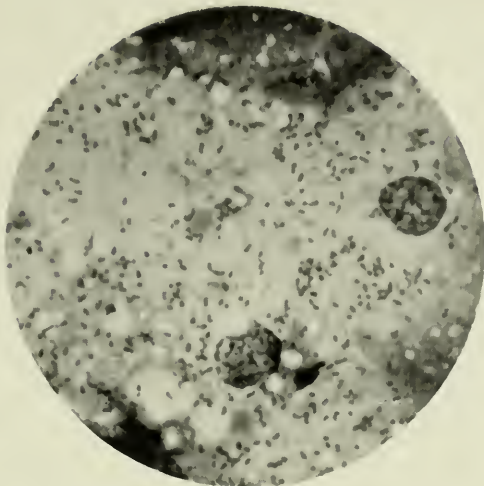


Fig. 4

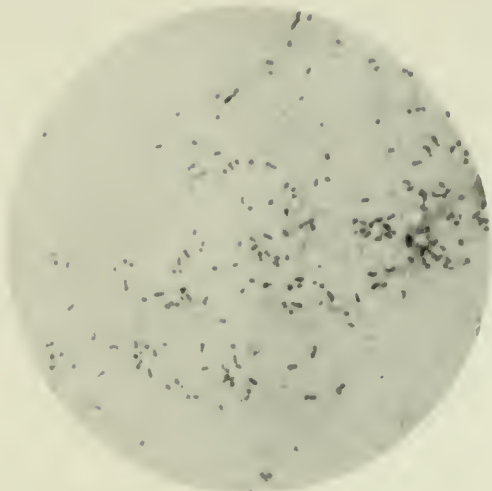


Fig. 5

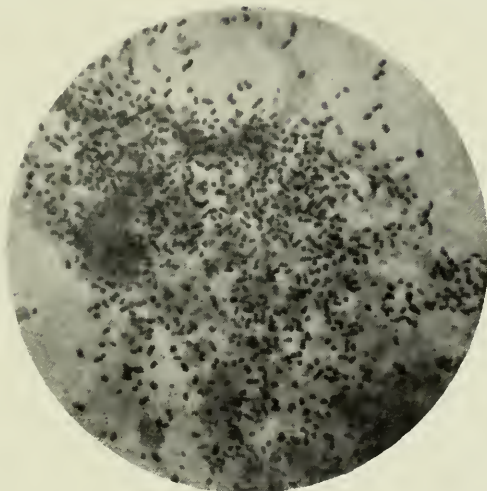


Fig. 6

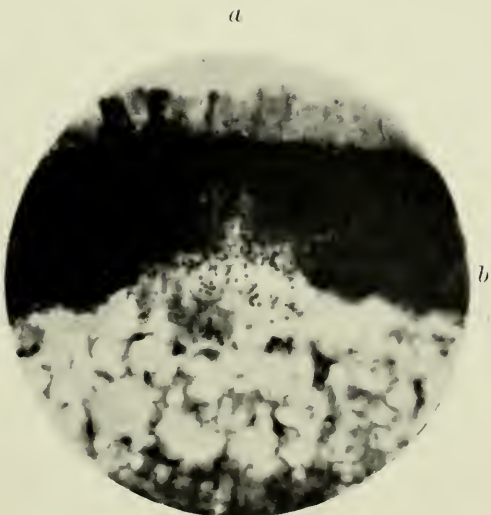


Fig. 7

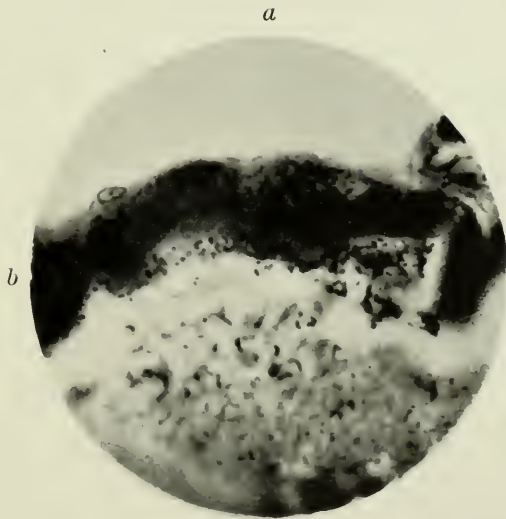


Fig. 8





THE INFLUENCE OF THE AGE OF PARENT AT BIRTH  
OF OFFSPRING UPON THE DEVELOPMENT OF EYE  
COLOUR AND INTELLIGENCE—A CORRECTION.

By R. J. EWART, M.D., D.Sc.

OWING to some regrettable slips in the arithmetical work of the paper dealing with eye colour and intelligence recently published in this *Journal*<sup>1</sup> several of the constants are inaccurate. The whole of the calculations have now been re-worked and the correct values are given below. I have incorporated some fresh material, the analysis of which somewhat strengthens the conclusions already published.

I shall first discuss the new material. Table I records the eye colour distributions of infants aged from one to six months by categories of parental age and the resultant correlation. Although the estimated probable error, derived by the formula appropriate to product moment correlations, is not a complete measure of the random fluctuations of such coefficients as these, the relation found would appear to be of some significance and is actually the largest yet obtained from material of this class.

An obvious criticism is that the age distribution of the infants may be materially different for different arrays of parents. To test the importance of this, an extended series of observations was made upon children, from birth

Table I.

*Influence of Age of Parent at Birth and Eye Colour of Young Infant  
(one to six months of age). (Barking.)*

Age of Parent	Eye colour. Infant.			Percentage not blue
	Blue	Not blue	Total	
16th to 20th years	16	0	16	0
21st „ 24th „	59	6	65	9.2
25th „ 28th „	65	13	78	16.1
29th „ 32nd „	55	12	67	17.9
33rd „ 36th „	34	7	41	17.1
37th „ 40th „	35	12	47	25.5
41st „ 44th „	10	2	12	16.6
Totals	274	52	326	
$r = +.206 \pm .036.$				

<sup>1</sup> *Journ. of Hygiene*, xvi. 1917, pp. 12-35.

*Influence of the Age of Parent*

to the age of one year, the material being derived from an infant clinic (Tables II-IV). The resulting constants are:

Standard Deviation, Mother's Age	...	...	...	1.5894 ± .0386
" " Child's "	...	...	...	2.9995 ± .0726
Correlation of Child's Eye Colour and Mother's Age	...	...	...	.115 ± .035
" " " " Child's "	...	...	...	.348 ± .030
" " Age " Mother's "	...	...	...	.034 ± .035

Hence the partial correlation of mother's age and child's eye colour. Child's age constant

$r = .110 \pm .034.$

Table II.

*Child's Age and Mother's Age.*

Child's age in weeks	Mother's age in years								Totals
	16-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	
1-4	3	11	11	8	5	5	2	—	45
5-8	7	23	20	26	15	14	5	1	111
9-12	3	12	15	8	9	11	1	—	59
13-16	2	7	10	7	4	6	2	—	38
17-20	2	1	14	8	4	3	1	—	33
21-24	—	6	6	6	4	3	—	—	25
25-28	1	5	4	2	3	2	2	—	19
29-32	1	7	5	2	3	3	—	—	21
33-36	—	—	—	—	1	3	—	1	5
37-40	—	2	3	1	2	—	—	—	8
41-44	—	—	2	1	—	—	1	—	4
45-48	—	2	1	4	2	—	—	—	9
49-52	1	1	1	2	2	2	—	—	9
	20	77	92	75	54	52	14	2	386

Table III.

*Child's Age and Eye Colour.*

Child's age in weeks	Child's eye colour		
	Blue	Not blue	Total
1-4	43	2	45
5-8	103	8	111
9-12	51	8	59
13-16	24	14	38
17-20	23	10	33
21-24	21	4	25
25-28	14	5	19
29-32	19	2	21
33-36	2	3	5
37-40	4	4	8
41-44	3	1	4
45-48	4	5	9
49-52	7	2	9
	318	68	386

Table IV.

*Child's Eye Colour and Mother's Age.*

Age of mother in years	Child's eye colour		
	Blue	Not blue	Total
16-20	20	—	20
21-24	66	11	77
25-28	74	18	92
29-32	60	15	75
33-36	45	9	54
37-40	39	13	52
41-44	12	2	14
45 and over	2	—	2
	318	68	386

It appears therefore that some weight must be assigned to the objection offered but that when it is allowed for correlation persists.

In Table V the age of the father is taken into consideration. Information on this point being obtained indirectly through the mother, it is doubtless not very accurate. The correlation, of the same order of magnitude as found for the mother and child, rather suggests that any biological significance attaching to the previous results is not dependent upon intra-uterine nutritive changes.

Table V.

*Age of Father at Birth of Child. (Children under 1 year when observed.)*

Father's age in years	Children's eye colour		
	Blue	Not blue	Total
16-20	1	—	1
21-24	29	6	35
25-28	64	12	76
29-32	67	12	79
33-36	37	11	48
37-40	42	13	55
41-44	19	5	24
45-48	15	4	19
49-52	4	1	5
53-56	1	1	2
57-60	2	—	2
	281	65	346

Standard Deviation, father's age, 1·849.

Coefficient of correlation,  $r = \cdot 0924 \pm \cdot 036$ .

I now pass to the correction of previously published results. The numbering of the tables is that of the paper cited.

*Middlesbrough School Children (younger).*

		Boys	Girls
Tables VIII and VII.	Age of parent at birth and eye colour ...	$r = \cdot 138 \pm \cdot 032$	$\cdot 048 \pm \cdot 032$
„ X and IX.	Age of parent at birth and child's eye colour ... ..	$r = \cdot 149 \pm \cdot 032$	$\cdot 071 \pm \cdot 032$
„ XI and XII.	Eye colour, mother and child ... ..	$r = \cdot 524 \pm \cdot 024$	$\cdot 623 \pm \cdot 019$



*Influence of the Age of Parent*

*Making the Third Factor Constant in Each Case.*

	Boys	Girls
Tables VIII and VII.	$r = .070 \pm .033$	$.046 \pm .032$
„ X and IX.	$r = .092 \pm .032$	$.053 \pm .032$
„ XI and XII.	$r = .514 \pm .020$	$.622 \pm .019$

*Barking School Children (older).*

Table II.	Eye colour, mother and child...	...	...	...	$r = .608 \pm .020$
„ III.	Age of parent at birth and child's eye colour	...	...	...	$r = .005 \pm .032$
„ IV.	Age of parent at birth and her own eye colour	...	...	...	$r = .009 \pm .034$

*Making the Third Factor Constant in Each Case.*

Table II.	$r = .607 \pm .020$
„ III.	$r = .001 \pm .034$
„ IV.	$r = .008 \pm .032$

*Random Observation.*

Table V.	Eye colour of any mother with any child but its own	...	...	$r = .030 \pm .072$
„ V.	Age of parent at birth with eye colour of any child but its own	...	...	$r = -.068 \pm .026$

*Adult Life.*

Table XIII.	Age of grandmother at birth of mother and mother's eye colour	...	...	$r = .003 \pm .022$
Table XIV.	Represents Tables VII and VIII taken together and refers to mother's eye colour only, that is, "Age at birth and her own eye colour"	...	...	$r = .093 \pm .022$

Taking the chief constants we have:

1.	Young infants	...	...	$r = .206 \pm .036$
2.	Young school children, boys	...	...	$r = .092 \pm .032$
	„ „ „ girls	...	...	$r = .052 \pm .032$
3.	Older school children	...	...	$r = .001 \pm .034$
4.	Adults	...	...	$r = .003 \pm .022$

From these figures it is seen that the value of  $r$  obtained in samples of a young infant population disappears or becomes very small in sampling older children. In the younger school children the correlation for girls is not significant, for boys it may be so and therefore may indicate some bias. Hence I suggest that the rate of change of eye colour is more rapid in the later born, and that of the two sexes, the boys are probably somewhat later than the girls in reaching the full development of pigmentation.

If the development of shade, from the primitive blue of birth, is studied, it would seem that none of the ultimate colours can be regarded as transitional stages, hence if a scale could be devised, blue must occupy the middle position, the brown being towards one end and the grey towards the other. Or rather the primitive blue in the centre and the resultant colours occupying some position on the surface of a sphere. Hence there may be some justification for the division of eye colour into three groups, grey, blue and brown, and for treating them as though the distribution were Gaussian.

If this assumption is justifiable, then we may arrange our data in the following way, taking first the question as to whether there is a significant

difference in distribution of eye colour in those women who reproduce before and after the 30th year.

*Reproducing Women.*

Age	Brown	Blue	Grey, etc.	Totals
40 years and under at time of observation	246 (33·5)	234 (31·9)	253 (34·5)	733
41 years and over at time of observation... ..	181 (30·7)	161 (27·3)	247 (41·9)	589
	427	395	500	1322

30 years and under at time of birth, 40 years and under at time of examination.

Distance of brown from  $\bar{x} = \cdot 4245$

„ „ grey „  $\bar{x} = \cdot 3988$   
 $\cdot 8233$

Standard Deviation  $1\cdot 214 \pm \cdot 019$ .

31 years and over at time of birth, 41 years and over at time of examination.

Distance of brown from  $\bar{x} = \cdot 5046$

„ „ grey „  $\bar{x} = \cdot 2035$   
 $\cdot 7081$

Standard Deviation  $1\cdot 412 \pm \cdot 023$ .

From this it is seen that there is a slight and perhaps significant difference between the two groups, dependent upon a diminution in the number classed as blue and a large increase amongst the greys. But it must be remembered that the range of age in the latter class is from 41 years to 58 years, and the former from 28 years to 40 years, and that some change is to be expected on that account.

It seems reasonable therefore to suppose that so far as these observations allow, there is no definite evidence of selection with respect to eye colour in a population of reproducing women.

Turning to the school children:

*School Children. All ages from 7 years.*

Boys and Girls. Eye Colour.

	Brown	Blue	Grey, etc.	Totals
Born at age of 30 years and under	357 (28·4)	582 (46·3)	318 (25·3)	1257
„ „ 31 „ „ over	269 (31·1)	340 (39·3)	256 (29·6)	865
	626	922	574	2122

30 years and under.

Distance of brown from  $\bar{x} = \cdot 5715$

„ grey „  $\bar{x} = \cdot 6653$   
 $1\cdot 2368$

Standard Deviation  $\cdot 809 \pm \cdot 013$ .

31 years and over.

Distance of brown from  $\bar{x} = \cdot 4934$

„ grey „  $\bar{x} = \cdot 5361$   
 $1\cdot 0295$

Standard Deviation  $\cdot 9713 \pm \cdot 020$ .

At least *prima facie*, the distribution deviates from the parental type and in the opposite direction so far as the relation of age to the proportion classed as brown is concerned. I do not, however, desire to put much weight upon constants deduced on an assumption which is somewhat arbitrary.

It has occurred to me that the existence of correlation when parental age is correlated with the eye colours of young children and its evanescence when older children are involved may be a reflection of a phenomenon suggested by earlier results, viz. that the variability of filial arrays increases with the parental age at birth. Given a surface of zero regression but with increasing array variability, truncations of it should exhibit correlation. This may be illustrated in the special case of Gaussian arrays.

Suppose that all  $x$  arrays of  $y$  are Gaussian and further that every  $\bar{y}_x = \bar{y}$ . Then the correlations of the two surfaces formed by dividing the original surface by a plane intersecting the axis of  $y$  at right angles in the line  $y = 0$  are equal and opposite if  $\sigma_{y_x}$  increases with  $x$ .

Let  $\bar{x}_1, \sigma_{x_1}, \bar{x}_2, \sigma_{x_2}, \bar{y}_1, \sigma_{y_1}, \bar{y}_2, \sigma_{y_2}$  be the means and standard deviations of the two halves, all measurements being from the means.

$$\begin{aligned}\text{Then} \quad \bar{x}_1 &= \bar{x}_2 = \bar{x} = 0, \\ \sigma_{x_1} &= \sigma_{x_2} = \sigma_x, \\ \bar{y}_1 &= -\bar{y}_2, \\ \sigma_{y_1} &= \sigma_{y_2}.\end{aligned}$$

Consider the contribution made to the sum product  $S_1xy$  by the array  $y_{x=-s}$ . It is

$$-\frac{s\alpha_{-s}}{\sqrt{2\pi}\sigma_{y_{x=-s}}} \int_{-\infty}^0 ye^{-\frac{y^2}{2\sigma_{y_{x=-s}}^2}} dy = \frac{s\alpha_{-s}\sigma_{y_{x=-s}}}{\sqrt{2\pi}},$$

where  $\alpha_{-s}$  is a function of  $x = -s$ .

Similarly the contribution of the array  $y_{x=+s}$  is

$$-\frac{s\alpha_{+s}\sigma_{y_{x=+s}}}{\sqrt{2\pi}}.$$

Thus the contribution of corresponding arrays is

$$\frac{s}{\sqrt{2\pi}} (\alpha_{-s}\sigma_{y_{x=-s}} - \alpha_{+s}\sigma_{y_{x=+s}}),$$

and the complete product

$$\frac{1}{\sqrt{2\pi}} Ss (\alpha_{-s}\sigma_{y_{x=-s}} - \alpha_{+s}\sigma_{y_{x=+s}}),$$

for all values of  $s$ ; while the sum product of the other half is the same expression with signs reversed.

Hence the correlations are equal and opposite.

Consequently a fraction of the whole surface would exhibit correlation absent from a fair sample of the whole surface. Evidently the comparison of



a sample of young children with one of older children is not a simple case of truncation such as here contemplated, but it seems to me possible that the principle operates.

*Intelligence.*

The corrected coefficients for intelligence are as follows. They differ considerably from those already given.

*Child in Fifth Year (entering school).*

Table						Partial coefficient
XX.	Age of mother at birth of child and her own standard	...	...	...	$r = \cdot 060 \pm \cdot 027$	$\cdot 079 \pm \cdot 027$
XXII.	Age of mother at birth and class of child	...	...	...	$r = \cdot 029 \pm \cdot 024$	$\cdot 024 \pm \cdot 028$
XXIV.	Standard of mother and class of child	...	...	...	$r = \cdot 074 \pm \cdot 024$	$\cdot 076 \pm \cdot 027$
XXVI.	Age of mother on leaving school and her own standard	...	...	...	$r = \cdot 435 \pm \cdot 022$	$\cdot 438 \pm \cdot 022$
XXVIII.	Age of mother on leaving school and class of child	...	...	...	$r = \cdot 010 \pm \cdot 028$	$-\cdot 023 \pm \cdot 028$
XXX.	Age of mother on leaving school and age at birth	...	...	...	$r = -\cdot 031 \pm \cdot 028$	$-\cdot 063 \pm \cdot 027$

*Child in Thirteenth Year.*

Table						Partial coefficient
XXI.	Age of mother at birth of child and her own standard	...	...	...	$r = -\cdot 1119 \pm \cdot 038$	$-\cdot 139 \pm \cdot 042$
XXIII.	Age of mother at birth and standard of child	...	...	...	$r = \cdot 110 \pm \cdot 041$	$\cdot 155 \pm \cdot 042$
XXV.	Standard of mother and standard of child	...	...	...	$r = \cdot 349 \pm \cdot 037$	$\cdot 389 \pm \cdot 036$
XXVII.	Age of mother on leaving school and her own standard	...	...	...	$r = \cdot 415 \pm \cdot 035$	$\cdot 430 \pm \cdot 034$
XXIX.	Age of mother on leaving school and standard of child	...	...	...	$r = \cdot 017 \pm \cdot 047$	$-\cdot 146 \pm \cdot 047$
XXXI.	Age of mother on leaving school and age at birth	...	...	...	$r = -\cdot 082 \pm \cdot 042$	$-\cdot 016 \pm \cdot 043$

The main points are, firstly, that the correlation between the standard of the mother on leaving school at a constant age with the class of the child in its 5th year is significant but very small, and the correlation between the standard of mother at constant age with standard of child at 13th year is very much larger, but not as large as might be expected. This may be due to errors of record or to the fact that the mental characters upon which scholastic intelligence depends, hardly exist at the 5th year and are not even fully developed at the 14th year.

If this explanation is adopted as correct, then intelligence falls into the same category as eye colour, that is to say, at the 5th year the scholastic intelligence of a child corresponds to the eye colour of a new born babe, and hence there is no significant correlation between age of mother at birth and class of child. At the 14th year we are dealing with a period during which intelligence is only half developed, that is to say, our record corresponds to eye colour during the first year and hence a significant correlation is found. Had our record been one dealing with a period of life when intelligence is as fully developed as it ever will be, then in all probability the correlation

would become insignificant. This assumes that intelligence follows the same lines as the other characters investigated, viz., that the chief effect of age of the uniting germ cells is to produce an increased variability in those upon which the time influence is the greater.

Still the difference between the value of the association between age of mother at birth and her own standard for the 5th year being + .08 and for the 13th — .14, definitely suggests that the two series differ in other ways, beyond the fact that the families concerned contain a child in the 5th–13th years. As was previously stated the data are not above suspicion of bias.

REPORT OF BACTERIOLOGICAL INVESTIGATION OF  
TETANUS CARRIED OUT ON BEHALF OF THE WAR  
OFFICE COMMITTEE FOR THE STUDY OF TETANUS.

By W. J. TULLOCH, M.D., R.A.M.C., Bt. Major.

Lecturer in Bacteriology, University of St Andrews, Member of the War Office Committee  
for the Study of Tetanus.

(With 17 Diagrams.)

(From the Laboratories of the R.A.M. College and the Lister Institute  
of Preventive Medicine.)

CONTENTS.

	PAGE
Letter to Major-General Sir David Bruce, K.C.B., F.R.S., A.M.S., Chairman of War Office Committee for the study of Tetanus . . . . .	104
Introduction . . . . .	106
I. Types of <i>B. tetani</i> obtained from wounds of men suffering from tetanus	107
(a) Fallacies of investigation . . . . .	107
(b) Tabulation of cases due to various types . . . . .	109
(c) Discussion of findings . . . . .	113
II. Types of tetanus bacilli recovered from men showing no evidence of tetanus . . . . .	114
III. The results obtained in Section I and in Section II are contrasted .	117
Note on geographical distribution of the various types of <i>B. tetani</i> on the Western Front . . . . .	117
IV. Demonstration of antibodies, other than agglutinins, specific to the serological types of <i>B. tetani</i> . . . . .	120
(a) Preparation of sera . . . . .	121
(b) Preliminary investigation of sera . . . . .	122
(c) Technique of phagocytic tests . . . . .	124
(d) Results obtained in making phagocytic tests . . . . .	125
(e) Anti-phagocytic property of whole culture . . . . .	128
(f) Examination of toxin to determine whether it is leucotoxic .	131
V. Investigation of mechanism of infection in tetanus . . . . .	135
(a) Does <i>B. tetani</i> , in addition to elaborating a spasm-producing substance, also give rise to a tissue debilitating poison? .	137
(b) As tissue debilitating influences certainly assist in the develop- ment of tetanus infection, is there any particular debilitating influence that is of special significance in this connection? .	138
(c) As concomitant infection with certain organisms stimulates the growth of spores of <i>B. tetani</i> in tissue, is it not possible that the converse may be equally true? May not some concomitant infections reduce the toxogenic capacity of <i>B. tetani</i> ? .	142



	PAGE
(d) Preliminary experiments conducted with a view to the elaboration of a method for studying infection with <i>B. tetani</i> . . . . .	147
(e) Experiments dealing with prophylaxis with anti-tetanic serum . . . . .	150
(f) Investigation of anti-infective properties of antitoxic and anti-bacterial sera . . . . .	155
VI. Investigation of dressings in relation to anaërobic infections of wounds . . . . .	172
(a) Methods used . . . . .	172
(b) Inherent fallacies . . . . .	174
(c) Does presence of anaërobes in wounds seriously interfere with the process of healing? . . . . .	175
(d) Is there any method of treatment commonly used by the Army Surgeon abroad which tends to eliminate anaërobe infection? . . . . .	176
(e) Influence of dressings in use in Home Hospitals, etc. . . . .	178
(f) Rapidity with which healing takes place under various dressings . . . . .	179
Influence of dressings used abroad upon the rate of healing . . . . .	181
Influence of dressings used in Home Hospitals . . . . .	182
(g) Influence of excision . . . . .	184
(h) Examination of wounds of long standing . . . . .	186
(i) Examination for presence of <i>B. tetani</i> in a series of 100 wounds . . . . .	187
(j) Examination for presence of <i>B. Welchii</i> in a series of 100 wounds . . . . .	188
VII. Can agglutination of stock emulsions of <i>B. tetani</i> by the serum of patients suffering from early tetanus be employed as a method for diagnosis and for determining "Type of Infection" in tetanus? . . . . .	196
VIII. Relation of serological type of bacillus isolated, to haemagglutinating type of men from whom obtained . . . . .	196
IX. A note on two cases of abdominal tetanus . . . . .	196
X. Presence of <i>B. tetani</i> in the faeces of men returned from overseas compared with those from civilian faeces . . . . .	197
XI. Synopsis and general conclusions . . . . .	198

To Major-General Sir DAVID BRUCE, K.C.B., F.R.S.,  
Chairman of the War Office Committee for the Study of Tetanus.

SIR,

I have the honour to present the following Report dealing with the bacteriological research which has been prosecuted on behalf of the Tetanus Committee, during the period October 1917–October 1918.

Most of the work herein reported has been carried out jointly by Miss D. M. Cayley and myself.

Miss Cayley is solely responsible for Section VI (pp. 172–195) of the Report, in which is discussed the influence which various dressings and surgical procedures exert upon the anaërobic flora of wounds. The work was arduous and particularly trying, in that the information gained was by no means commensurate with the effort entailed in obtaining it. Miss Cayley is therefore to be congratulated upon her assiduous attention to this task.

The Report is divided into the following Sections:

I. An inquiry into the occurrence of the various types of *B. tetani* in the wounds of men suffering from tetanus.

II. An inquiry into the occurrence of the various types of *B. tetani* in wounds of men showing no evidence of tetanus.

III. A discussion of the facts set forth in Sections I and II.

IV. Experiments carried out *in vitro* to determine whether immune sera, prepared by inoculation of *whole culture* into animals, contained antibodies, other than agglutinins, specific to the "Types."

V. Experiments conducted *in vivo* to examine the problem of *infection* with *B. tetani*, as contrasted with *intoxication* due to absorption of the products of that organism.

The points dealt with in Section V are:

(a) The importance of a suitable *nidus* for the development of infection.

(b) Relation which the degree and nature of the tissue destruction or debilitation necessary for the inception of infection bears to the development of tetanus, and to prophylaxis by antitoxin.

(c) The part played by concomitant infection with micro-organisms other than *B. tetani* in activating or depressing the infective process and the intoxication in tetanus.

(d) The immunity conferred by the use of *mono-typical antitoxic* and *anti-bacterial* sera.

VI. The influence which various surgical procedures exert upon infection of wounds due to anaërobic bacteria.

VII. Attempts (a) to diagnose tetanus, and (b) to determine the "Type" of the infection in cases of the disease by means of an agglutination reaction, using the blood of patients suffering from tetanus for agglutinating stock emulsions of *B. tetani*.

VIII. An inquiry into the question of whether the serological Types are evolved as a result of the residence of the bacillus in the tissues of an individual belonging to one or other of the haemagglutinating groups of men.

IX. A discussion of two cases of abdominal tetanus.

X. The results of examination of faeces for the presence of *B. tetani* in the intestinal contents of

(a) Civilians,

(b) Men returned from active service.

Throughout each phase of the investigation one object only has been kept in view—the possible application of laboratory findings to the improvement of prophylaxis and therapeutics of tetanus.

Until *mono-typical antitoxic* and *anti-bacterial* sera are available in much larger quantities than is possible when ordinary laboratory animals are used for serum preparation, many of the questions raised by the work described in this Report must remain unsettled.



The investigations discussed in this Report are therefore admittedly incomplete, but the work has, I think, reached a phase in which it may, with advantage, be submitted for discussion.

I have the honour to be, Sir,

Your obedient Servant,

(Signed) WILLIAM J. TULLOCH, M.D., Bt. Major, R.A.M.C.

Lecturer in Bacteriology, The University of St Andrews.

#### INTRODUCTION.

IN a paper published in the *Journal of the R.A.M.C.*, December 1917, it was demonstrated, that *B. tetani* was susceptible of classification by serological methods into at least three "Types." Since the publication of that paper, a fourth Type has been encountered; but, so far, it has been demonstrated only in five instances—four being from cases of declared tetanus which were not, however, fatal.

The serological classification of *B. tetani* at once suggested the question: "What relationship, if any, do the various Types of the bacillus bear to the causation and pathology of the disease?"

The necessity for an inquiry was insistent, as both the prophylaxis and therapeutics of tetanus might have to be modified in view of the findings obtained.

The investigation was prosecuted as follows:

(a) As many cases of tetanus as possible were examined by bacteriological methods, in order to determine the serological Type of the bacillus responsible for the causation of the disease in each instance.

(b) In order to control these findings, the frequency with which the various serological Types of *B. tetani* could be obtained from wounds of men not suffering from tetanus, was made the subject of inquiry.

(c) Laboratory experiments, both *in vitro* and *in vivo*, were carried out, in order to determine whether crossed immunity to intoxication or to infection with the various Types, or their products, indicated that the typing of the bacilli was or was not of importance in relation to the pathogenesis of the disease and its serum prophylaxis. The question of the degree of passive immunity to *infection* conferred by the injection of antitoxin, naturally called for examination in this connection.

(d) The influence which various surgical procedures might have in preventing or lessening mass infection with anaërobcs was also investigated. This Section of the work is of special interest and importance, as the most important element in the prevention of anaërobe infections is the surgical procedure employed in the treatment of wounds.

(e) The question was investigated as to whether diagnosis of the disease in its prodromal stage might not be assisted by an agglutination



technique, in which stock emulsions of the Type bacilli were exposed to patients' sera.

(f) The relationship which concomitant infection with certain of the commoner anaërobes—other than *B. tetani*—might bear to the causation of tetanus, was also made the subject of inquiry.

## SECTION I.

### TYPES OF *B. TETANI* OBTAINED FROM WOUNDS OF MEN SUFFERING FROM TETANUS.

This Section of the investigation was undertaken with a view to determining what influence, if any, universally applied serum prophylaxis exerted upon the incidence, course, and issue of the disease, in respect of the serological Types of the bacillus responsible for its causation.

From examination of cultures, seven in number, obtained from various Serum Institutes, it was found that all seven specimens conformed serologically to the "U.S.A. standard culture." As these seven cultures comprised those commonly in use in the English Serum Institutes for the preparation of tetanus antitoxin, it seemed not improbable, that serum, corresponding to one serological type of the bacillus only, was being employed for prophylaxis, so far as the bulk of our own troops was concerned. This might result in the elimination of the disease produced by one serological Type of the bacillus, but might not, to the same extent, reduce the incidence of the disease, when the causal organism was *heterologous* to the prophylactic serum.

This appeared to be strikingly borne out by the examination of the first 25 strains of *B. tetani* obtained from cases of the disease, as typed by the agglutination method. Only one belonged to the same serological group as the standard U.S.A. culture—hereafter referred to as "Type I." At the same time, of the four cultures of toxic tetanus bacilli that had been then isolated from wounds of men not suffering from the disease, three conformed to that Type on serological investigation.

It will be seen from the further consideration of this Section of the Report, that although the hypothesis tentatively advanced above is possibly correct, the demarcation between cases due to Type I bacilli and cases due to the other Types among inoculated men, is not so clear cut as the earlier inquiries might have led one to believe.

#### (a) *Fallacies of investigation.*

In making this investigation, the number of fallacies which may be introduced is such, that any figures obtained and quoted can only have a relative value. Apart from the technical difficulties which have to be overcome in attempting to obtain the organism in a sufficient state of purity to permit of the application of serological tests, other quite uncontrolled sources of error

affect the problem. Great difficulty is, therefore, experienced in interpreting the results obtained.

The present series of cases, 100 in number, represents the successful results obtained in the examination of about 200 specimens. It may seem that a 50 per cent. rate of successful investigations is low, but it must be borne in mind, that the material was not collected by the bacteriologist, and that it was forwarded to the laboratory often from long distances. The investigator was therefore not in a position to obtain optimum results.

Among the cases in which I failed to obtain suitable growths for agglutination, a small number of swabs grew no organisms at all; a larger number grew only aërobes, and in a still larger number there were no organisms resembling *B. tetani*, either in meat-water cultures or in cultures made in media of a more complex composition. A considerable number of the cultures which did develop bacilli bearing a spherical terminal spore, failed to agglutinate in presence of any of the four Type sera. The majority of these agglutinated in presence of a serum prepared by inoculating an animal with a culture of a non-toxic round end-sporing bacillus, whose precise position and relationship have not yet been determined.

In point of fact, it is remarkable, in view of the technical difficulties that are encountered, that not so few, but so many as 50 per cent. were successfully investigated. This purely technical difficulty then, introduces an experimental error, the assessment of which is by no means easy. This error can only be reduced by examination of a further series of cases. In addition to the difficulty of isolation, the occurrence in the cultures of a peculiar inagglutinable phase renders statistical inquiry, based on agglutination, a laborious and not completely satisfactory procedure.

I wish here to emphasise a point that might otherwise be misunderstood, viz., that I do not consider it probable that all organisms capable of elaborating spasm-producing toxins, will necessarily be agglutinated by one or other of the four Type sera; I do think it probable, however, that the majority of such organisms can be so agglutinated.

In any series of cases of tetanus, the incidence, course, and termination of the disease are all modified by a number of factors. These factors, which in each individual case will modify the disease, are:

(i) The nature of the wound, the extent of the solution of continuity, the involvement of muscle or other structures, the degree of devitalisation of tissue due to direct trauma, the degree of interference with the nutrition of the part due to concomitant trauma to vessels, etc.; all these must play a part, and an important part, in the pathogenesis and final issue of any case. Short of visiting and personally examining every case, the part played by each of these factors in the causation of the disease cannot be even remotely estimated.

(ii) The position of the wound and its anatomical relationship may have some bearing on the incidence and result of infection with *B. tetani*.



(iii) The nature and extent of concomitant infection with other organisms is an extremely important, and a quite uncontrollable (uncontrollable from the standpoint of the investigator) factor in determining the onset of tetanus. This question is dealt with to some extent in Section V of the Report.

(iv) As a natural corollary of (a) and (c) *supra*, the nature of the first surgical interference carried out immediately after the reception of the wound, and, to a less extent, the procedure thereafter followed, will influence profoundly the subsequent history of a case in respect of the incidence of all specific infection—in the present instance infection due to gas gangrene bacilli and *B. tetani*.

(v) The conditions under which the wound was received, notably, the length of time the man lay out before he was picked up, will also have a bearing upon the subsequent development of tetanus and other infections.

(vi) Other conditions, which cannot be dismissed without consideration in this connection, are meteorological, geographical, geological, seasonal and agricultural conditions.

With so many potential sources of error intrinsic to the cases and inseparable from the technique that one is forced to adopt, the present series of 100 cases cannot be expected to give unequivocal information on the point of issue. None the less, the series is of interest and suggests certain not unimportant considerations.

(b) *Tabulation of cases due to various types.*

Table I shows the number of cases, the incubation period, and the final result in those instances, in which Type I bacilli were recovered from wound exudates of cases of declared tetanus.

Table II shows the same with regard to Type II bacilli.

Table III shows the same with regard to Type III bacilli.

Table IV shows the same with regard to Type IV bacilli.

Table I. *Cases of Tetanus due to Type I.*

Recovery or death	Prophy- lactic	Thera- peutic	Local	General	Where wounded	Onset
1. Recovered	+	+	...	+	St Quentin ...	7 days
2. Recovered	+	+	...	+	Delville Wood ...	7 „
3. <i>Died</i>	+	+	...	+	Vieux Berquin ...	7 „
4. <i>Died</i>	—	+	...	+	Peronne ...	8 „
5. Recovered	+	+	...	+	... ..	9 „
6. <i>Died</i>	—	+	...	+	Hull Docks ...	9 „
7. Recovered	+	+	...	+	France ...	9 „
8. Recovered	+	+	...	+	Delville Wood ...	10 „
9. <i>Died</i>	+	+	...	+	Near Maillet ...	10 „
10. Recovered	+	+	+	...	Posièrès ...	11 „
11. <i>Died</i>	+	+	...	+	Somme ...	11 „
12. Recovered	+	+	+	...	Bon Hamel ...	11 „
13. <i>Died</i>	+	—	+	...	Amiens ...	12 „
14. Recovered	+	+	+	...	Thiepval ...	13 „
15. Recovered	+	+	...	+	Somme ...	13 „





Table III. *Cases of Tetanus due to Type III.*

Recovery or death	Prophy- lactic	Thera- peutic	Local	General	Where wounded	Onset
1. <i>Died</i>	+	+	...	+	Vimy Ridge ...	3 days
2. <i>Died</i>	+	+	...	+	France ...	5 "
3. <i>Died</i>	+	+	...	+	Cambrai ...	6 "
4. Recovered	+	+	...	+	Villers Bretonneux	6 "
5. <i>Died</i>	+	+	...	+	Thiepval ...	7 "
6. <i>Died</i>	-	+	...	+	Cambrai ...	8 "
7. Recovered	+	+	...	+	Albert ...	8 "
8. <i>Died</i>	-	+	...	+	Yorkshire ...	8 "
9. Recovered	+	+	...	+	Bullecourt ...	9 "
10. <i>Died</i>	+	+	...	+	... ...	10 "
11. Recovered	+	+	+	...	Amiens ...	10 "
12. Recovered	+	+	+	...	Ypres ...	11 "
13. <i>Died</i>	+	+	...	+	Borsinghe ...	12 "
14. Recovered	+	+	...	+	... ...	13 "
15. <i>Died</i> *	+	+	...	+	Villers Bretonneux	14 "
16. Recovered	+	+	...	+	Bullecourt ...	14 "
17. <i>Died</i>	+	+	...	+	Trones Wood ...	14 "
18. Recovered	+	+	...	+	Trones Wood ...	15 "
19. <i>Died</i>	+	+	...	+	St Quentin ...	16 "
20. Recovered	+	+	...	+	Passechendaele ...	17 "
21. Recovered	+	+	+	...	Passechendaele ...	18 "
22. Recovered	+	+	...	+	Ypres ...	18 "
23. <i>Died</i>	+	+	...	+	... ...	19 "
24. Recovered	+	+	...	+	Amiens ...	20 "
25. <i>Died</i>	+	+	...	+	Somme ...	30 "
26. Recovered	+	+	...	+	Gonnellieu ...	30 "
27. <i>Died</i>	+	+	...	+	Guinchy ...	43 "
28. Recovered	+	+	...	+	Blighty Wood ...	54 "
29. Recovered	+	+	...	+	Albert ...	57 "
30. Recovered	+	+	...	+	Guyencourt ...	65 "
31. Recovered	+	+	...	+	France ...	81 "
32. Recovered	+	+	...	+	Cambrai ...	101 "
33. <i>Died</i>	+	+	...	+	... ...	149 "

\* Prophylactic inoculation made late.

Table IV. *Cases of Tetanus due to Type IV.*

Recovery or death	Prophy- lactic	Thera- peutic	Local	General	Where wounded	Onset
1. Recovered	-	+	+	...	Posières ...	4 days
2. Recovered	+	+	...	+	Villers Pouchée ...	17 "
3. Recovered	+	+	...	+	Bapaume ...	48 "
4. Recovered	+	+	...	+	Ypres ...	49 "

The findings described in Tables I, II, III, and IV, are graphically summarised in the following Diagram I (p. 112).

Commenting on these results, I wish to call particular attention to a point of great importance, viz., that the prophylactic administration of A.T.S., although failing to prevent absolutely the occurrence of tetanus, tends very markedly to reduce the death-rate from the disease. This is indicated by the fact that death occurred in six out of seven cases of generalised tetanus in

the present series, which did *not* receive a prophylactic dose of A.T.S. Whereas of 75 cases, which were definitely known to have received a prophylactic dose of serum, only 25 terminated fatally.

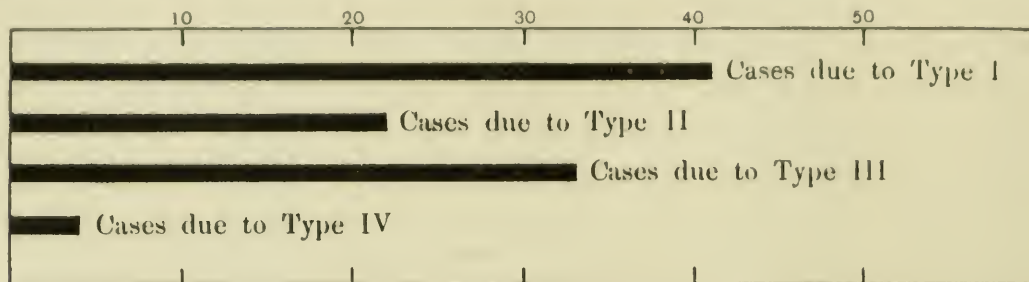


DIAGRAM I. Types of *B. tetani* obtained from 100 cases of tetanus in which the organism was cultured in a sufficient state of purity to permit of serological examination.

If now, we limit ourselves to those cases which received a prophylactic injection of serum the following figures are obtained.

(a) Type I appeared to be the organism responsible for the disease in 38 cases; six deaths occurred in this series, of which four were definitely attributable to tetanus, one was certainly not due to that disease, and in the remaining case the cause of death was doubtful. This gives a death-rate of 13.1 per cent. for cases due to Type I.

(b) Type II was found in 18 cases, six of which terminated fatally. In five instances death was definitely attributable to tetanus—a death-rate for Type II of 27.7 per cent.

(c) Of Type III there were 31 cases with 12 deaths, all of which were attributable to tetanus. In one case the prophylactic dose of A.T.S. was known to have been administered late. Therefore, we may regard the death-rate as 11 out of 31 (= 35.5 per cent.).

(d) Of Type IV infection there were only four cases none of which were fatal.

These facts are presented graphically in Diagram II.

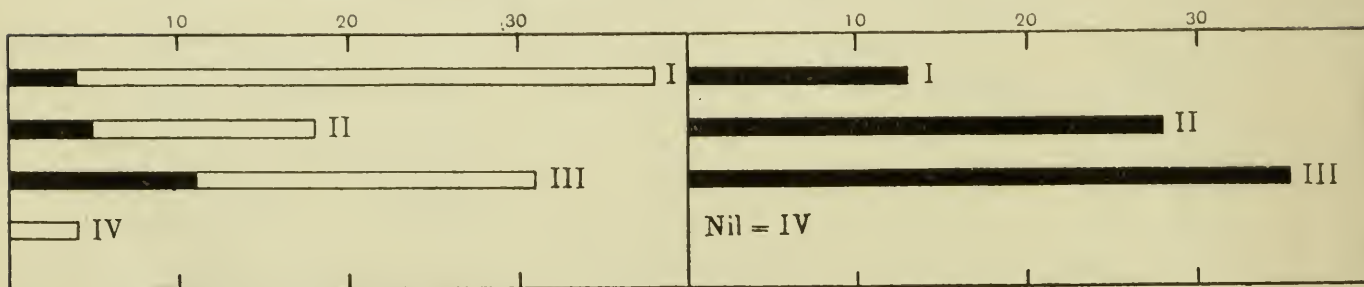


DIAGRAM II. Number of each type of *B. tetani* recovered from declared cases of the disease occurring in inoculated men.

The actual number of deaths due to each type

= ██████

Deaths from each type of infection expressed as a percentage of the number of cases in which each type was "isolated" from declared cases of tetanus in inoculated men.

These findings call for the following comments.



(c) *Discussion of findings.*

(a) Owing to the total number of cases examined (100) being small, it is difficult to draw definite conclusions from the results obtained. I think, however, that the findings are suggestive, particularly in view of the fact that, both in the case of Type II and in that of Type III infections, the death-rate is considerably higher than when Type I is the organism responsible for the disease.

(b) Although Type I bacilli appear to be responsible for a larger number of cases of tetanus than are Types II or III, the death-rate among inoculated men from Type I is lower than when Type II or Type III is the infecting organism.

This might be due simply to there being a relatively larger number of strains of Types II and III virulent to man than there are of Type I; on the contrary, it may mean that the serum used for prophylaxis affords more adequate protection against infection with Type I bacilli than against infection with Types II and III. Further Sections of the present Report deal with attempts which have been made to determine which of these hypotheses is correct.

The figures, so far obtained, are, however, susceptible of inquiry from another point of view. If the low death-rate and (as will be seen from Section III of the present Report) the relatively low rate of incidence of cases due to Type I infection, are attributable to special qualities of the serum used for prophylaxis, then, the number of cases occurring during the first two weeks after wounding, and the proportion of those which terminated fatally, should be relatively greater in infections due to Types II and III than in those due to Type I.

On examining Tables I, II and III, it will be noted that:

1. Of 38 cases of Type I infection in inoculated men the onset of the disease occurred within 14 days in 15 instances, and death was attributable to tetanus in three out of 15 instances.

2. Of 17 cases in inoculated men in which the causal organism was a Type II bacillus, eight occurred within the 14-day period and of these three proved fatal.

3. Of Type III cases there are 31 in inoculated men; in 15 instances the onset occurred within the 14-day period and eight of them proved fatal. Death was attributable to tetanus in all eight cases.

These results may, for purposes of comparison, be graphically expressed thus.

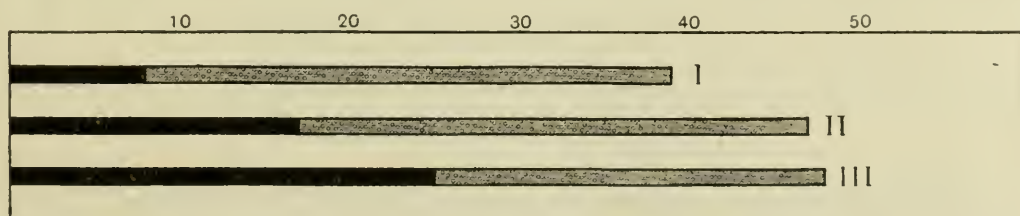


DIAGRAM III. Cases of tetanus in inoculated men due to Types I, II, and III in which onset occurred within 2 weeks. Rates expressed as percentages.

▨ = Incidence.    ■ = Death rate.

Regarded superficially, these figures do not appear to carry conviction, as the numbers dealt with are too small.

A more careful consideration of them, however, indicates that they are extremely suggestive, and a fairly definite interpretation of these figures is made possible by the results obtained in Sections III and V of this Report. Section III shows that Type I bacilli are found much more frequently than are Types II or III in wounds of (inoculated) men who show no evidence of tetanus; while Section V demonstrates that, in passively immunised animals, the development of tetanus infection depends to a preponderating extent upon the degree of tissue destruction occurring at the time of injury. It is only natural then, that Type I cases can and will occur within the 14-day period, and that a certain number of these will prove fatal; for, if the degree of tissue destruction exceeds certain limits, no amount of antitoxin—in practical dosage at least—will prevent the occurrence of the disease.

That the findings obtained in this Section of the work represent a fair average of what would probably be found in examining a larger series of cases is suggested by the fact, that, of about 100 cases of tetanus occurring in inoculated men, from whose wounds I failed to obtain *B. tetani* in a sufficient state of purity to permit of its serological examination, the death-rate was 21 per cent., whereas in the series under discussion, the death-rate is much the same—25 per cent.

#### GENERAL CONCLUSIONS FROM SECTION I (pp. 107–114).

An examination of 100 cultures of *B. tetani* obtained from wounds of men suffering from tetanus shows:

1. That the value of serum prophylaxis is very great indeed. Practically all the cases of the disease which occur in men who have not received serum prophylaxis terminate fatally.

2. There are indications both from

- (a) The higher death-rate, and

- (b) The earlier onset of the disease among men who did receive serum prophylaxis, that, either, Types II and III are more virulent for man than is Type I, or, the protection afforded by the serum at present in use, is more adequate against infection due to Type I bacilli than to Types II or III.

#### SECTION II.

##### TYPES OF TETANUS BACILLI RECOVERED FROM MEN SHOWING NO EVIDENCE OF TETANUS.

An investigation into the distribution of the various serological Types of *B. tetani* in wounds of men who show no clinical evidence of tetanus is an essential corollary to the previous inquiry. If such an investigation be not made, wrong interpretations might be put upon the findings of Section I,



and a false perspective might be obtained of the actual incidence of the various Types of the bacillus in wounds. During the period in which the series of cases discussed in Section I was being submitted to bacteriological examination, a number of swabs were forwarded to the laboratory from wounds in which organisms resembling *B. tetani* had been demonstrated by other investigators, although the patients showed no evidence of tetanus.

These were submitted to complete bacteriological examination along with cultures containing bacilli resembling *B. tetani* which Miss Cayley encountered in making an investigation into the anaërobic flora of a series of 100 wounds.

In all, 25 such strains were obtained from wounds. These 25 strains were each carefully examined and only qualified as members of one or other of the serological Types of *B. tetani* when they

- (1) agglutinated in presence of one or other of the agglutinating sera,
- (2) produced toxin when grown *in vitro*, or,
- (3) caused tetanus when injected together with a tissue-debilitant.

It may seem peculiar that a differentiation is made between

- (a) power to produce toxin when grown *in vitro*, and
- (b) power to produce the disease when washed spores of the culture are injected together with a tissue-debilitant.

But, however, such differentiation of these two factors must be made, in view of the findings which are discussed *in extenso* in Section V of the present Report.

Of these 25 strains which were proved both by agglutination and animal experiment

19	were	Type I	bacilli
3	„	„	II „
2	„	„	III „
1	was	„	IV „

The results obtained in Section II are graphically shown in the following diagram—Diagram IV.

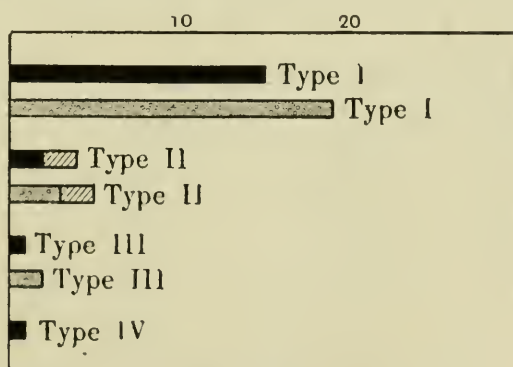


DIAGRAM IV. Actual numbers of *B. tetani*, proved by animal tests and obtained from wounds of men showing no evidence of tetanus.

- = From series of 100 wounds.
- = From all sources.
- ▨ = Result equivocal.



The details of the examination of each of these strains are given in the following Table.

Table V.

A. *Strains of B. tetani obtained from the examination of 100 wounds of men not suffering from Tetanus.*

No.	Animal experiment	Agglutination	Since wounding	Where wounded
1.	Toxin expt. positive	Type I	7 days	Ypres
2.	" "	" I	7 "	"
3.	" "	" I	20 "	Albert
4.	Toxin expt. negative Infection expt. positive	" I	24 "	"
5.	Toxin expt. positive	" I	25 "	Villers Bretonneux
6.	" "	" I	30 "	Mericourt
7.	Toxin expt. negative Infection expt. positive	" I	32 "	Cambrai
8.	Toxin expt. negative Infection expt. positive	" I	35 "	"
9.	Toxin expt. negative Infection expt. positive	" I	39 "	"
10.	Toxin expt. positive	" I	64 "	Battersea
11.	" "	" I	112 "	Ypres
12.	" "	" I	130 "	Cambrai
13.	See Footnote (i)	" I	733 "	Hohenzollern Redoubt
14.	Toxin expt. positive	" I	860 "	Poelcapelle
15.	" "	" I	882 "	Cambrai
16.	" "	" II	15 "	Bapaume
17.	See Footnote (ii)	" II	100 "	Cambrai
18.	Toxin expt. positive	" II	138 "	Passehendaele
19.	See Footnote (ii)	" II	54 "	Monchy
20.	Toxin expt. positive	" III	16 "	Orvillers
21.	" "	" IV	305 "	Hermies

B. *Strains of B. tetani obtained from wounds of men not suffering from Tetanus obtained from various other sources.*

1.	Toxin expt. positive	Type I	49 days	Cambrai
2.	" "	" I	56 "	"
3.	" "	" I	56 "	"
4.	" "	" I	No details obtainable	
5.	" "	" II	8 days	No further details
6.	" "	" III	No details obtainable	

*Footnote (i).* With reference to No. 13, toxin experiments were negative, and when an attempt was made to carry out the infection experiment, the animals died of gas gangrene.

*Footnote (ii).* Culture 17 which agglutinated in presence of Type II serum was lost before the animal experiments were completed; culture 19 is still under investigation.

If we limit ourselves to the consideration of those cultures obtained by Miss Cayley in the examination of 100 wounds in Table I A, it is found that

Type I bacilli were found in 15 cases

„	II	„	„	2	„
„	III	„	„	1	case
„	IV	„	„	1	„

In addition to the two cultures of Type II recorded above there were the two other cases, namely cultures 17 and 19, which could not be fully investigated.

The results dealt with in this Section will be more fully discussed in Section III.

Commenting on these results it is seen:

1. That Type I bacilli appear to be much more frequently found in wounds than are bacilli of the other serological Types.

2. That, in 100 wounds of men who showed no evidence of tetanus, *B. tetani* could be recovered in certainly 19 and probably in 21 instances.

### SECTION III.

THE RESULTS OBTAINED IN SECTION I AND SECTION II ARE CONTRASTED.

NOTE ON THE GEOGRAPHICAL DISTRIBUTION OF THE VARIOUS SEROLOGICAL  
TYPES OF *B. TETANI* ON THE WESTERN FRONT.

If the findings of Section I, which deals with the investigation of swabs from wounds of men suffering from tetanus, be contrasted with the findings of Section II, which deals with the demonstration of tetanus bacilli in the wounds of men showing no evidence of tetanus, the following results are obtained.

The total number of tetanus bacilli isolated from cases of the disease = 100.

The total number of tetanus bacilli isolated from wounds of men showing no evidence of tetanus = 25.

Type I from cases	= 41	41 %
„ I „ indifferent wounds	= 19	76
„ II „ cases	= 22	22
„ II „ indifferent wounds	= 3	12
„ III „ cases	= 33	33
„ III „ indifferent wounds	= 2	8
„ IV „ cases	= 4	4
„ IV „ indifferent wounds	= 1	4

This is graphically shown in Diagram V.

*Note.* In Diagram V, the open column referring to Type II indicates the percentage of proved Type II bacilli obtained from wounds of men not suffering from tetanus.

The hatched column indicates the Type II cultures from the same source in which the investigation is not completed.

These findings also suggest that the protection afforded by the serum at present in use is more adequate in respect of Type I infection than in respect of infections due to Types II and III. The number of Type IV cases is as yet too small to permit of any deductions being made from the results obtained.

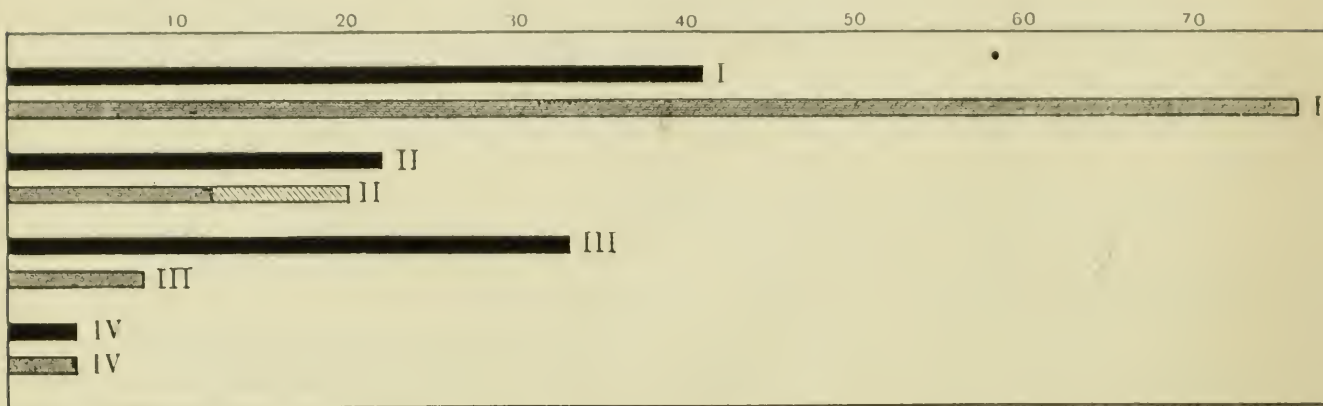


DIAGRAM V. Types of *B. tetani* "isolated" from cases of the disease compared with those "isolated" from "indifferent" wounds each expressed as percentage of the total numbers "isolated" from each source.

■ = From cases.

▒ = From "indifferent" wounds.

▨ = Type II bacilli from "indifferent" wounds the examination of which was not completed or the result obtained was equivocal.

The figures so far obtained may be dealt with from another standpoint. We know that 20 per cent. of wounds may contain tetanus bacilli, it is also known that the incidence of tetanus among the wounded in England is roughly 1 per 1000. From these figures it appears that in 1000 wounds there are 160 which are infected with Type I bacilli, and therefore, only 1 in 160 infections with that organism gives rise to declared tetanus.

On the same basis 1 in 20 at the lowest, or 1 in 40 at the highest estimate, gives rise to the disease in the case of Type II infections. Of Type III infections there appear to be only 10 per 1000, and therefore, every tenth man infected with Type III bacilli may fall a victim to the disease.

It is questionable if this reasoning is strictly justifiable, for the assumption is made that three cases instead of one have occurred per 1000 wounded men. As the results, however, are only of comparative and not of absolute significance, the same error is introduced in each instance.

The suggestion which arises from the consideration of the facts from this point of view may be diagrammatically shown thus (Diagram VI, p. 119).

The following conclusions appear, therefore, to be permissible from the comparison of the results of the investigations dealt with in Sections I and II.

1. That Type I bacilli are of more frequent occurrence in nature than are Types II and III.



2. That the serum at present in use for prophylaxis affords more adequate protection against Type I infection than against infection with the other Types.

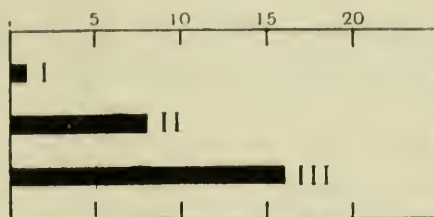


DIAGRAM VI. The columns indicate that of 160 men infected with

Type I	bacilli,	1	would	take	the	disease
Type II	„	8	„	„	„	„
Type III	„	16	„	„	„	„

These deductions are made with reserve, as so many factors enter into the causation of the natural disease in man, and so many more appear to affect the toxogenicity of *B. tetani*. Short of unequivocal experimental evidence being obtained, that more adequate protection is afforded by a serum *homologous* to the infecting bacillary Type, than by a serum *heterologous* thereto, one cannot arrive at a definite conclusion concerning the point at issue.

#### GEOGRAPHICAL DISTRIBUTION OF VARIOUS TYPES OF *B. TETANI* ON THE WESTERN FRONT.

As a matter of scientific interest, if not of practical importance, a record was kept of the districts in France where each man received his injury. In 97 instances this information was available. If the area occupied by British troops be divided roughly into two districts, (1) north of the La Bassée Canal, and (2) south of La Bassée Canal, it is found that:

(i)	of 50 strains of Type I bacilli	13	came from the N Area				
		37	„	„	S	„	
(ii)	„ 15 „ „ II „	11	„	„	N	„	
		4	„	„	S	„	
(iii)	„ 27 „ „ III „	6	„	„	N	„	
		21	„	„	S	„	
(iv)	„ 5 „ „ IV „	1	„	„	N	„	
		4	„	„	S	„	

These figures suggest that the various serological Types may have different geographical distribution in view of the relatively large number of Type II bacilli which were obtained from Flanders. This is further borne out by the fact that during the period of the Flanders offensive Type II were frequently found, while with the alteration of the fighting to the Somme area, Types I and III were those commonly obtained.

It is only fair to note that among a small number of cultures obtained from men who received injuries in this country all types of *B. tetani* were represented.

## SECTION IV.

DEMONSTRATION OF ANTIBODIES, OTHER THAN AGGLUTININS, SPECIFIC TO THE SEROLOGICAL TYPES OF *B. TETANI*.

While the previous Sections of this Report suggest that the incidence of tetanus among inoculated men may to some extent depend upon the type of the infecting bacillus, and the serological relationship which it bears to the organism used for the preparation of prophylactic antitoxin, the findings by no means prove that there is such a relationship. In the foregoing Sections stress has been laid upon the difficulty of interpreting the results obtained by making a statistical inquiry into the problem, and these difficulties are again here emphasised.

It was necessary therefore to prosecute experimental investigation while the other phases of the work were proceeding.

A. *The first question which naturally arises is:* "Is the toxin of one serological Type of the bacillus more adequately neutralised by its own *specific* antitoxin than by antitoxin produced by inoculation of the product of a serologically *heterologous* bacillus?"

The findings of Leuchs (*Zeitschrift für Hygiene*, 1918, Bd 65, p. 55) in respect of the toxins of different strains of *B. botulinus* suggested that quantitative differences, at least, might exist between the soluble toxins of the various Types in relation to the neutralising value of an antitoxin corresponding to any one Type.

Experiments were therefore made with a view to examining this point. The results obtained showed definitely:

(a) That no *qualitative* difference existed between the toxins produced by the bacillus of the different Types.

One antitoxin neutralised the toxin of any Type or of all Types.

(b) That, if a *quantitative* difference exist, it is so slight, that when mice or rats are the animals employed for making these tests, no quantitative relationship of a specific nature can be demonstrated.

B. *The second question which therefore arises is:* "As the soluble toxins of all Types of *B. tetani* are the same, but, as the bacilli themselves are different one from another as *antigens*, is this *antigenic* difference of the bacilli of any import in their pathogenic property, and is it of special importance in the unnatural conditions arising from universally applied serum prophylaxis?"

It seemed not improbable, that the value of antitoxin for prophylactic purposes might depend upon more than one factor:

(1) We know that one factor is the capacity for neutralising the *spasm-producing* toxin of all tetanus bacilli.

(2) Hypothetically, another factor that might be considered is the capacity for preventing *infection*.



The former of these factors we have demonstrated to be non-specific in respect of the Types—at least in so far as the experimental animals employed are concerned. The second (hypothetical) factor, which has not hitherto been inquired into in a systematic way, may be specific. If this be the case, a *mono-typical* serum would protect most adequately against infection with a serologically *homologous* organism.

The inquiry therefore resolved itself into the investigation of the following problems:

(a) Can antibodies other than “agglutinins,” which are *specific* to the Types, and “anti-spasmins” which are *not specific* to the Types, be evoked by inoculation of cultures of the bacilli or of their products?

(b) If such specific antibodies can be demonstrated, and serum containing them be proved to be of prophylactic value, how should we proceed to prepare sera containing these antibodies?

*Note.* The term “proved to be of prophylactic value” means, proved to prevent *infection* which will cause death in the unprotected animal or in the animal inadequately protected by antitoxin.

(c) What relationship do the various demonstrable antibodies bear to one another?

(d) What is the mechanism of tetanus *infection* as opposed to tetanus (spasm-producing) *intoxication*?

To investigate these questions two separate and distinct methods of inquiry were followed:

(1) *In vitro* experiments were carried out with various sera and cultures.

(2) Infection experiments *in vivo*.

This Section of the Report (No. IV) deals only with the *in vitro* experiments.

#### (a) *Preparation of sera.*

Nine rabbits were immunised as follows:

I received a course of immunisation with the filtered toxin of a *two-day culture* of Type I bacilli.

The toxin was stored for six weeks in the ice-chest before use, and a *two-day toxin* was chosen so that the least possible autolysis of the bacillus had occurred, and yet an adequate toxin was obtainable.

II was immunised by inoculating intravenously *whole culture* of Type I bacilli grown for 24 hours.

III underwent a course of immunisation with *washed* Type I bacilli.

I' corresponded to I but Type II *toxin* was used

II'           ,,           II       ,,       II *culture* was used

III'          ,,          III     ,,          II *washed bacilli* used

I''           ,,           I        ,,           III *toxin* was used

II''          ,,          II        ,,          III *culture* was used

III''         ,,         III     ,,         III *washed bacilli* used.



The rationale of choosing the three methods of immunisation was that:

(a) The animals inoculated with toxin would produce antibodies to the filtrable antigens, and, as stored toxin was used, only antibodies to those antigens which were stable.

(b) The animals immunised with all the products of a young culture would produce antibodies to:

(i) any spasm-producing toxin that might have developed,

(ii) to the bacilli themselves,

(iii) to any soluble but unfiltrable or unstable antigens which the organism might develop during its phase of active growth.

It might be argued that it is improbable that a soluble but unfiltrable antigen would be elaborated during the growth of any organism; but if the filtration experiments dealing with the products of pathogenic anaërobic bacilli in general be scrutinised, one notes that difficulty has been experienced owing to the large amount of active product that is absorbed in filtration, and filtrates deficient in toxicity have been obtained.

Young cultures were designedly chosen for immunising animals II, II', II'', because it is known, that certain of the pathogenic anaërobic bacilli—notably *B. Welchii*—elaborate tissue-debilitating poisons during their period of active growth. These poisons are evanescent, and the toxicity of cultures markedly declines on continued incubation for several days at 37° C.

It seemed possible that *B. tetani* might give rise to similar aggressive products that have not so far been demonstrated, because they were not sought for.

(c) The animals inoculated with *washed* cultures should produce only antibodies to the bacillary substance.

These nine animals, after having been under immunisation for approximately two months, were bled by Durham's technique to the extent of about 10 c.c. each and a complete series of observations was made with the sera so obtained.

#### (b) Preliminary investigation of sera.

Firstly, the antitoxic titre was roughly determined.

Rabbit No. I—approximately 12 units per c.c.

„	II	„	2	„	„
„	III	„	1 unit	„	„

*Note.* Animal No. III, owing to an accident, received a number of inoculations with bacilli that had been washed in saline only once, while No. III' and III'' were immunised with bacilli which had been washed several times.

No. I' approximately 5 units per c.c.

„	II'	„	2	„	„
„	III'	„	less than 0.5 per c.c.		
„	I''	„	12 units per c.c.		
„	II''	„	2	„	„
„	III'	„	less than 0.5 per c.c.		

Secondly, the agglutinin titre of each was determined, and cross experiments with all three Types of bacilli were carried out.

This Section of the work is of considerable importance for two reasons:

(i) A perfectly just criticism might be made of a previous communication on the serological differentiation of tetanus bacilli *inter se* (*Journal of R.A.M.C.*, Dec. 1917), viz. that owing to the low titre of the serum then

Table VI.

Serum No. I=Type I Toxin							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Type I	—	—	—	—	—	—	—
„ II	—	—	—	—	—	—	—
„ III	—	—	—	—	—	—	—
Serum No. II=Type I 24-hour Whole Culture							
Type I	+	+	+	+	+	+	—
„ II	—	—	—	—	—	—	—
„ III	—	—	—	—	—	—	—
Serum No. III=Type I Washed Bacilli							
Type I	+	+	+	+	+	—	—
„ II	—	—	—	—	—	—	—
„ III	—	—	—	—	—	—	—

---

Serum No. I'=Type II Toxin							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Type I	—	—	—	—	—	—	—
„ II	—	—	—	—	—	—	—
„ III	—	—	—	—	—	—	—
Serum No. II'=Type II 24-hour Whole Culture							
Type I	—	—	—	—	—	—	—
„ II	+	+	+	+	+	+	—
„ III	—	—	—	—	—	—	—
Serum No. III'=Type II Washed Bacilli							
Type I	—	—	—	—	—	—	—
„ II	+	+	+	+	+	+	—
„ III	—	—	—	—	—	—	—

---

Serum No. I''=Type III Toxin							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Type I	—	—	—	—	—	—	—
„ II	—	—	—	—	—	—	—
„ III	—	—	—	—	—	—	—
Serum No. II''=Type III 24-hour Whole Culture							
Type I	—	—	—	—	—	—	—
„ II	—	—	—	—	—	—	—
„ III	+	+	+	+	+	—	—
Serum No. III''=Type III Washed Bacilli							
Type I	—	—	—	—	—	—	—
„ II	—	—	—	—	—	—	—
„ III	+	+	+	+	+	+	—

employed—1/400—the specific agglutination results therein figured might give a false impression concerning the complete specificity of the Types of *B. tetani* from the standpoint of agglutination. The low titre of the serum might unduly enhance the specificity of the tests.

(ii) The cultures used for inoculating these animals had been isolated a year previously, and, since isolation, had been frequently sub-cultured—sometimes daily over long periods.

I call attention to this, because it had been suggested that serologically different races of the bacillus might be evolved as a result of residence in human tissue or in close proximity thereto.

Table VI illustrates the agglutination reactions obtained with these sera in presence of emulsions of the Type bacilli.

The results of this titration of agglutinins show that:

(a) The *agglutinin* titre is no index of the *antitoxic* value of the serum: this of course is what would naturally be expected.

(b) Even when sera of high agglutinin titre are prepared, the agglutination reactions remain *specific*.

(c) The stock Type cultures have remained true to Type for a period exceeding 12 months.

(d) The agglutinin response is quite as marked when *washed cultures* are used for immunisation as when *whole cultures* are used for this purpose.

*Thirdly, the influence which these various sera exhibited in stimulating phagocytosis of Type bacilli was made the subject of inquiry.*

#### (c) *Technique of phagocytic tests.*

1. Washed white corpuscles (human) were prepared exactly according to the method advised by Wright; but in place of using a leucocyte layer in making the tests, the deposit of whole blood obtained after the final centrifugalisation was well mixed, and the thick blood cream so obtained was employed.

2. Unwashed actively growing 24-hour cultures served for the bacillary suspension. In order to obtain a sufficiently active growth, these cultures were made in trypsinised broth standardised to be alkaline to  $\alpha$ -naphthol-phthalein but acid to phenol-phthalein, and enriched by addition of fresh tissue.

3. The reaction was carried out at 38° C. and incubation proceeded for 15 mins. The mixtures of the various reagents were made and incubated in small agglutination tubes instead of in capillary tubes. This permits of the reagents being well distributed by shaking.

4. Each serum to be examined was tested in a series of dilutions according to the method of Neufeld, and all the sera were fresh when tested.

5. Each mixture consisted of:

- 1/50 c.c. culture,
- 1/50 „ of serum—usually diluted,
- 2/50 „ of washed blood cream.



6. After incubation, films were made in the ordinary way, fixed for two minutes in a mixture of equal parts of saturated aqueous solution of  $\text{HgCl}_2$  and absolute alcohol, and were stained for two minutes with 1/10 carbol-fuchsin.

7. Examination of 100 consecutive leucocytes in each preparation was then made. I employed at first a 1/12 objective with a No. 4 ocular for making the counts, but subsequently found that, owing to the size of the bacillus, this could be done quite satisfactorily with 1/6 objective and a No. 4 ocular.

8. The method of Neufeld was adopted, as group relationship between the bacilli might be encountered. Dilution of the serum would to some extent overcome this difficulty.

9. No attempt was made to count the number of organisms taken up by each leucocyte; but, in order to obtain a relative figure, the leucocytes which contained considerable numbers of bacilli were regarded as positive cells, while those containing no bacilli, or only one or two, were considered negative.

Thus, in Table VII and subsequent Tables, the figures given represent the percentage of leucocytes in each preparation which took up the bacilli with avidity. It was remarkable how rarely one met with a cell containing few organisms; the leucocytes were either filled with bacilli or contained none. Difficulty of interpretation did not therefore arise.

(d) *Results obtained in making phagocytic tests.*

The results of these phagocytosis experiments are shown in Table II.

Table VII. *The figures relate to the percentage of leucocytes taking up large numbers of bacilli.*

VII. A.				
Serum No. I = Type I toxin				
Dilutions of serum				
Culture used	No serum control	1/20	1/40	1/80
Type I	3	5	6	2
„ II	0	0	0	0
„ III	0	0	0	0
Serum No. II = Type I 24-hr. whole culture				
Type I	×	85	92	84
„ II	×	10	6	1
„ III	×	77	14	15
Serum No. III = Type I washed bacilli				
Type I	×	71	48	33
„ II	×	0	0	0
„ III	×	17	9	0

× = Not done.

*Note.* In this and in subsequent tables relating to phagocytic experiments, the dilutions indicated at the head of each column give the dilution of serum originally made, so that in this instance, the actual dilutions in presence of which the test was carried out were 1/80, 1/160, and 1/320.

## VII. B.

Culture used	No serum control	Serum No. I' = Type II toxin		
		Dilutions of serum		
		1/20	1/40	1/80
Type I	1	0	2	2
„ II	0	0	0	0
„ III	0	0	0	0
Serum No. II' = Type II 24-hr. whole culture				
Type I	×	1	5	3
„ II	×	68	67	64
„ III	×	0	0	0
Serum No. III' = Type II washed bacilli				
Type I	×	5	1	4
„ II	×	50	31	37
„ III	×	0	0	0

## VII. C.

Culture used	No serum control	Serum No. I'' = Type III toxin		
		Dilutions of serum		
		1/20	1/40	1/80
Type I	0	0	2	1
„ II	0	0	0	0
„ III	2	2	0	0
Serum No. II'' = Type III 24-hr. whole culture				
Type I	×	87	89	85
„ II	×	28	16	5
„ III	×	99	98	100
Serum No. III'' = Type III washed bacilli				
Type I	×	2	2	1
„ II	×	4	1	0
„ III	×	44	26	4

*Note.* Experiments VII A and VII B were done together, using the same reagents, so that they are strictly comparable. My equipment did not permit of the simultaneous examination of all three, so that VII C was done on the following day.

The following points call for comment:

1. The *antitoxic* sera do not stimulate phagocytosis of the bacilli in presence of *whole culture*. It is to be noted, that the observations made with *antitoxic* sera serve as controls for the experiments with *anti-bacterial* sera.

2. The most active sera for stimulating phagocytosis are those prepared by inoculation of *whole culture*.

3. The sera prepared by inoculating *washed bacilli*, while giving an agglutinin titre as high as those prepared by inoculating *whole culture*, do not exhibit so high a phagocytic titre.

4. As regards the question of specificity, sera Nos. III, III', III'' (*i.e.* antisera to *washed bacilli*) exhibit this markedly in the above experiments, as also does No. II' (*i.e.* an antiserum to *whole culture* of Type II bacilli),

whereas Nos. II and II'', also *anti-whole* culture sera to Types I and III respectively, do not.

*Note.* Animal No. II' had been under immunisation for a shorter period than II and II''.

In view of the equivocal result obtained with sera Nos. II and II'', the experiment was repeated on the following day, but the dilutions of the sera were carried much further.

Serum No. III, owing to its having a demonstrable antitoxic content, 1 unit per c.c., was also included in this test, the results of which are shown in Table VIII.

Table VIII.

Cultures used	No serum control	Serum No. II = Type I 24-hr. whole culture				
		Dilutions of serum				
		1/50	1/100	1/200	1/400	1/800
Type I	0	89	63	50	4	0
„ III	0	5	2	0	0	0
Serum No. II'' = Type III 24-hr. whole culture						
Type I	×	37	13	4	0	0
„ III	×	98	78	36	0	0
Serum No. III = Type I washed bacilli						
Type I	×	24	14	2	0	0
„ III	×	1	0	0	0	0

*Note.* These experiments have been repeated many times and have given consistently specific results on each occasion. The experiment quoted (Table VII A and C) was *that one* of the series, which was most difficult to interpret.

I wish to call attention also to the discrepancy between the actual figures noted in Table VII, and those in Table VIII, in respect of the sera under examination; this appears to depend upon a variability of the anti-phagocytic properties of the cultures, or upon a variability of the phagocytic activity of the white cells from day to day, and renders futile any attempt that might be made to express these results in the form of an index of phagocytosis, unless a standard serum were made the basis of that index.

In order definitely to confirm the most important of the findings obtained in this series of investigations, viz. that *antitoxic* serum did not promote phagocytosis as did *anti-bacterial* serum, the experiment shown in Table IX was carried out.

Table IX.

	Serum No. I = Type I antitoxic		
	1/1	1/10	1/20
Type I	24	0	0
	Serum No. II = Type I 24-hr. whole culture		
Type I	79	82	84
	Serum No. I'' = Type III antitoxic		
Type III	34	0	2
	Serum No. II'' = Type III 24-hr. whole culture		
Type III	70	90	96



Normal serum controls 1/1 were included in the series, and gave substantially the same results as those obtained with 1/1 antitoxic serum.

In making this experiment, the technique already described was followed, but, to ensure that the employment of a 1/6 objective did not lead to avoidable error, the examination of the slides was made with a 1/12 semi-apochromatic lens with a No. 4 ocular, and care was taken critically to illuminate the preparation so that optimum conditions for microscopical examination were realised.

*Fourthly, the apparent anti-phagocytic property of whole culture was made the subject of investigation, and an attempt was made to determine whether toxin, as ordinarily prepared and stored, was also anti-phagocytic.*

(e) *Anti-phagocytic property of whole culture.*

Mechanism of the "anti-phagocytic property" of *whole culture*.

The results quoted in the previous Sub-section of this Report, suggest that the phagocytic activity of leucocytes in presence of anti-serum to *whole culture* may depend upon one or other of two things.

(i) That the serum prepared by inoculation of *whole culture* contains an antibody to an hypothetical aggressive antigen not present in *washed cultures*, and if present in filtrates, only in small quantity, *or*

(ii) That the *bacillary* substance, *per se*, is the antigen that determines the development of the property of stimulating phagocytosis in *anti-bacterial sera*.

It is not improbable that both factors, (i) and (ii), may be represented in the mechanism. By comparing Serum II with Serum III, II' with III', and II'' with III'', it is seen, that while the *anti-whole culture sera* are the more active in the presence of *whole culture*, yet the *anti-washed bacilli sera* are not altogether devoid of the power of inducing phagocytosis.

For the present then, leaving out the consideration of the question as to whether these antibodies which lead to the phagocytosis of tetanus bacilli are "stimulins," in the sense implied by Leishman, or "opsonins," in the sense implied by Wright, the point at issue was to determine if:

(a) *whole young culture* exhibited aggressive qualities not present in toxin obtained by filtration,

(b) these aggressive properties were specific to the serological Types of the bacilli, and could be countered by anti-bacterial serum prepared by inoculating *whole culture*.

The experiments quoted (Tables VII, VIII and IX, pp. 125-127) dispose of the question as to whether these aggressive properties can be neutralised by anti-bacterial (*whole culture*) serum.

To determine whether young *whole culture* did exhibit definite aggressive qualities, and at the same time to determine whether these were or were not specific to the serological Types, the following experiment was set up.

*Technique.*

Two centrifuge tubes of a 24-hour culture of Type I bacilli and two similar tubes of a 24-hour culture of Type III bacilli were centrifuged at high speed, in order to separate the bacilli from the products of their growth. The supernatant fluid from each tube was drawn off and kept separate. This procedure gave two tubes containing a deposit of Type I bacilli—tubes A and B—and two tubes containing a deposit of Type III bacilli—tubes C and D—and four tubes of supernatant fluid corresponding to these—namely A' and B', containing Type I supernatant fluids, and C' and D', containing Type III supernatant fluid.

The deposit in each of the four tubes was well shaken with saline and centrifuged. This procedure was repeated twice; after which the saline was finally drawn off with a pipette.

(1) To the deposit of washed Type I bacilli in A was added the supernatant fluid from a Type I culture—A'.

(2) To the deposit of washed Type I bacilli in B was added the supernatant fluid from a Type III culture—D'.

(3) To the deposit of washed Type III bacilli in C was added the supernatant fluid from a Type III culture—C'.

(4) To the deposit of washed Type III bacilli in D was added the supernatant fluid from a Type I culture—B'.

These four suspensions of bacilli were then exposed to washed white cells in presence of dilutions of Type I and Type III *anti-bacterial* sera. The results shown in Table X were obtained.

Table X.

Serum No. II=Type I 24-hr. whole culture					1/100	1/200
(a)	Type I whole culture	...	...	...	75	46
(b)	Type I bacilli + growth products of Type III	...	...	...	72	14
Serum No. II'=Type III 24-hr. whole culture						
(c)	Type I whole culture	...	...	...	11	4
(d)	Type I bacilli + growth products of Type III	...	...	...	30	8
Serum No. II=Type I 24-hr. whole culture						
(e)	Type III whole culture	...	...	...	6	7
(f)	Type III bacilli + growth products of Type I	...	...	...	31	18
Serum No. II'=Type III 24-hr. whole culture						
(g)	Type III whole culture	...	...	...	80	67
(h)	Type III bacilli + growth products of Type I	...	...	...	80	38

*Note.* The ordinary technique was followed in the above experiment, but the observations were made with a 1/12 objective and a No. 4 ocular, in order to exclude technical error as far as possible. In making the count, only those leucocytes containing no organisms were considered negative. This procedure had to be adopted in the present instance, as it was difficult to be sure that centrifugalisation had removed all the bacilli from the supernatant fluid.



Control experiments in which the supernatant fluids alone were exposed to the action of the *homologous* serum in presence of leucocytes showed that:

(a) When Type I supernatant fluid + Type I serum 1/100 + blood were incubated, 12 leucocytes were found to have taken up organisms.

(b) When Type III supernatant fluid + Type III 1/100 serum + blood were incubated, five leucocytes were found to contain organisms.

The real cause of the low counts obtained was, that there were so few bacilli present that many of the white cells never came into contact with bacilli, and the error therefore, due to the presence in these fluids of organisms that had not been deposited by centrifugalisation, was not very great.

Indeed, the error was more apparent than real, for there were so few bacilli present in these supernatant fluids that white cells containing more than two rods were seldom met with in the controls; while, in the above Table, the majority of the leucocytes considered as positive contained at least four or five bacilli.

In Table X the following points call for comment.

(i) When bacilli, supernatant fluid and serum are all *homologous*, consistently high counts are obtained with both dilutions of the serum—(a) and (g).

(ii) When bacilli and supernatant fluids are both *heterologous* to the serum, consistently low counts are registered with both dilutions—(c) and (e).

(iii) Where the bacilli are *homologous* to the serum and the growth products are *heterologous* thereto, a high count is obtained in presence of the higher concentration of the serum; but—as compared with the control, where *homologues* only are present in the mixtures—the lower concentration of the serum shows a relatively low phagocytosis—(b) and (h).

(iv) When the bacilli are *heterologous* to the serum and the growth products *homologous* to it, a relatively high count—as compared with the control in which both bacilli and growth products are *heterologous*—is obtained with the higher concentration of the serum—(d) and (f).

(v) The results obtained with *whole* cultures confirm that the reactions are specific to the Types.

From these observations the following deductions seem permissible.

(a) That the reaction to inoculation of *bacillary* substance alone, results in the development of antibodies which are specific to each Type and induce phagocytosis.

(b) From (iii) it may be deduced that while aggressive substances appear to exist in young cultures, these can, to a considerable extent, be neutralised by an *heterologous anti-bacterial* serum; it is only when the *heterologous serum* is diluted, that the specific aggressive quality becomes manifest.

(c) From (iv) the same deduction may be made. For it is seen that leucocytes, even when brought into contact with bacilli which are not *homologous* to the serum present in the mixture, show considerable phagocytic activity, provided that the bacterial products in which the bacilli are sus-



pended are homologous to the serum. This activity is naturally most marked in presence of the higher concentrations of serum, and appears to be specific in respect of the products of the various serological Types.

I do not wish to lay over much stress on this Section of the work knowing well the errors, both technical and subjective, that are liable to be introduced in such experiments.

The tests have been repeated and consistent results have been obtained so far as the method allows. Nevertheless, the evidence is by no means unequivocal. I submit, however, that it is highly suggestive, and indicates that there are at least three antigenic factors in cultures of *B. tetani*.

(i) The *spasm-producing toxin*, which is filtrable, and is non-specific in relation to the Types.

(ii) The *bacillary* substance, which is definitely specific to these Types.

(iii) A third *antigen*, which has anti-phagocytic properties, and appears to be specific to the Types.

These findings corroborate those which were obtained when comparative tests were made with *antitoxic*, *anti-whole-culture* and *anti-washed-bacilli* sera; indeed, the deductions which may be made from the one series of experiments are in complete agreement with those that may be made from the other.

*Finally, the question of whether toxin, as ordinarily prepared by filtration, exhibits anti-phagocytic properties, was made the subject of investigation.*

(f) *Examination of toxin to determine whether it is leucotoxic.*

In inquiring into whether tetanus toxin was leucotoxic, I made use of two methods of investigation.

The first series of tests was carried out thus:

Phagocytic tests were made with serum No. III'', which was prepared by inoculation of *washed* Type III bacilli—agglutinin titre 1/3200, antitoxic value < 0.5 unit per c.c., phagocytic titre low. The serum was diluted and exposed to various mixtures of reagents prepared thus:

Five tubes of a 24-hour culture of Type III bacilli were centrifugalised, and the deposit in each was retained. This deposit was washed by adding saline and again centrifugalised, the process being repeated twice. The supernatant saline, after the final centrifugalisation, was pipetted off so that a deposit of washed Type III bacilli was obtained in each of the five tubes—*a*, *b*, *c*, *d* and *e*.

(1) To deposit in (*a*) saline was added to the original volume.

(2) To deposit in (*b*) was added supernatant fluid obtained by centrifuging a 48-hour culture of Type III bacilli.

(3) To deposit in (*c*) was added the supernatant fluid from a 10-day culture of Type III bacilli.

(4) To deposit in (*d*) was added the toxin obtained by filtration of a 48-hour culture of Type III bacilli. This toxin had been stored in the ice-chest for three months before the test was made.

(5) To deposit in (e) was added the toxin obtained by filtration of a 10-day culture of Type III bacilli prepared six months before the test was made.

Phagocytic tests were then set up using each of these suspensions as the bacillary emulsions to be phagocytosed. The results shown in Table XI were obtained.

Table XI.

Serum No. III''=Type III washed bacilli						1/20	1/40	1/80
Washed bacilli	Type III	+ saline	...	...	...	46	18	9
"	"	+ supernatant fluid of	2-day culture			10	2	0
"	"	+	"	10	"	3	0	0
"	"	+ toxin from		2	"	12	2	1
"	"	+	"	10	"	39	13	2

These results show, that both the supernatant fluid and the toxin from the 2-day culture which was used, are definitely anti-phagocytic; while the toxin from the 10-day culture which was included in the test is not so.

It is to be noted that the 2-day toxin was not more lethal for mice than was the 10-day toxin.

With reference to the loss of anti-phagocytic potency in the case of the 10-day toxin, it is probable, that the prolonged storage rather than the prolonged incubation or the filtration, has been responsible for the decline; for, in respect of its anti-phagocytic power, it compares unfavourably both with the 2-day toxin and with the 10-day supernatant fluid.

As the phagocytic potency of Serum III''—*anti-washed-bacillary*—was known to be low, the experiment was repeated, using Serum No. II''—*anti-whole-culture*—the phagocytic potency of which was known to be high, and which also exhibited a demonstrable antitoxic titre. Using this serum the following results were obtained.

Table XII.

Serum No. II''=Type III 24-hr. whole culture						1/50	1/100	1/200
Washed bacilli	Type III	+ saline	...	...	...	27	73	61
"	"	+ supernatant fluid of	2-day culture			55	45	41
"	"	+	"	10	"	64	54	45
"	"	+ toxin from	2-day culture	...	...	47	44	28
"	"	+	"	10	"	66	65	60

The results shown in Tables XI and XII call for the following comments.

(a) The figures in Table XI leave no room for doubt that both supernatant fluids resulting from centrifugalisation of cultures of *B. tetani*, and certain toxins obtained by filtration, are anti-phagocytic. The questions of whether filtration reduces this leucotoxic factor, whether it stands storage, and at what period of growth it is present in largest quantity, or in most active condition, are not answered by the experiment quoted. In this instance—Table XI—it is possible, that owing to the low phagocytic potency of the







The figures indicate that *antitoxic* serum does neutralise the *anti-phagocytic* property of toxin. It may here be noted again, that the toxin from the 2-day culture is more anti-phagocytic than the toxin from the 10-day culture. As to whether the reaction is or is not specific in relation to the Types, the results obtained are equivocal; for the anti-phagocytic power of both the Type III toxins used in the test appears to be less than that of the Type II toxins.

The converse experiment was therefore carried out using a 1/5 dilution of Type III *antitoxic* serum and the results shown in Table XIV were obtained.

Table XIV.

Dilution of serum = 1/5, *i.e.* 1/30 of final mixtures.

Figures express percentage of leucocytes taking up many staphylococci.

				Serum diluted 1/5				
Toxin (undiluted)				1/50 c.c.	Staphylo. 1/50 c.c.	Blood 3/50 c.c.	Normal serum 1/50 c.c.	Type III antitoxin 1/50 c.c.
No toxin—saline only				...	...	...	86	×
Toxin from	2-day growth	Type III		..	..	..	10	63
..	10	..	III	..	..	..	41	83
..	2	..	II	..	..	..	22	34
..	10	..	II	..	..	..	38	83

*Note.* Experiments XIII and XIV were done on different days so that they are not strictly comparable.

Here again the *anti-phagocytic* property of toxin is apparent, and again, the toxin of the 2-day growth exhibits greater anti-phagocytic power than does that of the 10-day growth. Specificity appears to be fairly definite in this instance, at least so far as the toxin from 2-day growth is concerned. Thus, in the case of the *homologous* mixture, the number of active leucocytes is 63, as contrasted with 10 in the normal serum control. While, in the case of the *heterologous* mixture, it is 34 as compared with 22 for the corresponding control.

The results of these experiments are quite definite in respect of the following points.

- (i) Toxin is anti-phagocytic.
- (ii) The anti-phagocytic property of toxin can be neutralised by anti-toxin.
- (iii) If a specific relationship between toxin and antitoxin exist in this connection, the relationship is *quantitative*—the experiments quoted suggest such relationship.

In view of the variability of the anti-phagocytic power of the cultures and their products, a crucial experiment is difficult to perform. Although certain of the results then do suggest such a specific relationship, they fail to demonstrate that relationship unequivocally.

## RÉSUMÉ OF SECTION IV.

(I) *Antitoxic sera* do not stimulate phagocytosis of tetanus bacilli.

(II) *Anti-bacterial sera*, prepared by inoculation of *whole* culture, markedly stimulate phagocytosis; and the relationship between serum and organism is specific to the serological Types.

(III) Although *anti-bacterial sera*, prepared by inoculation of *washed* cultures, are active from the standpoint of agglutination, they are not so active in stimulating phagocytosis as are sera obtained by inoculation of *whole* culture.

(IV) The agglutinin titre, the antitoxic titre, and the phagocytic titre of the sera, are independent one of another.

(V) Tetanus toxin is apparently leucotoxic, but all specimens of toxin are not equally leucotoxic.

(VI) This leucotoxic factor can be neutralised by antitoxin; and the experiments quoted suggest that the neutralisation, by antitoxin, of this leucotoxic factor in toxin is specific in relation to the bacillary Types.

## SECTION V.

## INVESTIGATION OF MECHANISM OF INFECTION IN TETANUS.

## INTRODUCTION.

Section IV (pp. 120–135) of this Report shows definitely, that, apart from agglutinins, antibodies specific to the serological Types of *B. tetani* can be evoked by injecting *whole* cultures of that organism into animals. These findings are of considerable significance, but, unless it can be demonstrated *in vivo*, that anti-bacterial immunity plays some part in the prevention or cure of tetanus, their interest is largely academic.

In the first place, it must be clearly understood, that tetanus is due to *the growth* of *B. tetani* within, or in close proximity to, the tissues; and, that intoxication results only when sufficient growth of the organism has occurred. This emphasis of a palpable platitude is necessary, for, in the past, the attention of investigators has been so directed to the mechanism of intoxication in tetanus, that the conditions, which in nature must necessarily precede that intoxication, have been largely overlooked.

In this Section of the Report it is proposed to inquire into the mechanism of *Tetanus infection*, and to determine, if possible, whether the prophylaxis of the disease could be improved in any direction. This really involves the consideration of

- (i) Surgical measures.
- (ii) Concomitant infections, and how to deal with them.
- (iii) The possible value of anti-bacterial sera.



Before these problems could be examined, much preliminary work had to be done in order to elaborate a satisfactory technique for infecting the animals which would be used in the investigation. This presents considerable difficulty, for infection with *B. tetani*, and indeed, infection with any of the pathogenic anaërobes, is, at least in the early stage, saprophytic rather than parasitic in quality. Thus, it has long been known that inoculation of *washed* and *heated* tetanus spores does not frequently lead to the development of the disease, and it is only when a certain amount of tissue destruction has occurred that infection will "take." The same is true of infection with *Vibrio septique*; Besson has shown that spores of this organism may be injected into animals with no ill-effect; if, however, these spores be suspended in a fluid which has leucotoxic properties, infection invariably occurs. Emulsions of *B. Welchii* deprived of their growth products have also been injected without leading to infection, but when injections of the emulsions together with their growth products are made "trauma"—really due to presence of toxin—occurs, leading to the development of the organism.

These considerations have a direct bearing upon the causation of tetanus; for, concomitant infection with any pathogenic organism, by exerting a tissue-debilitating influence, may permit of the development of *B. tetani*. In natural infection of wounds with anaërobes, one cannot consider one infection alone, as every organism in the flora of the wound may exert an influence, stimulant or deterrent, upon the development of any one type of organism present. Wounds probably pass through a cycle of infections; certain aërobes and then certain of the more rapidly growing anaërobes appear in the first phases, to be followed subsequently by the more slowly developing bacteria including *B. tetani*. It must not be assumed, however, that all wounds will pass through the same cycle; for it will depend partly upon the relative numbers of the various organisms present in the infecting material, and partly upon peculiarities of the species or individual exposed to infection, which of the organisms present in the *inoculum* will develop first, and what sequence they will thereafter follow.

In the causation of tetanus the primary *necessary* factor is a certain degree of tissue destruction. In the nidus so formed, organisms grow, and among the earliest usually to appear are certain of the bacilli responsible for the causation of gas gangrene.

These organisms grow rapidly—especially *B. Welchii*—developing growth products which exert a necrotic action upon the tissues. This permits of the further growth of *B. tetani* and of other bacteria of specific infections. A vicious cycle is thus established, and the fact must not be lost sight of, that before even these adjuvant infections occur, it is essential that there should be tissue debility caused by direct trauma to the part, or trauma to important structures in relation thereto.

Therefore, the most important prophylactic measure which can be applied



for the limitation of anaërobic infections is, *adequate and enlightened surgical interference*, performed at the earliest possible moment after reception of the injury. Therefore wide resection of wounds, by removing devitalised tissue, prevents the establishment of this vicious circle.

This question, which is of paramount importance in the experimental work dealt with in this Section of the present communication, is more fully considered in Section VI, pp. 172–195.

These considerations prompted the following questions.

- (a) DOES *B. TETANI*, IN ADDITION TO ELABORATING A SPASM-PRODUCING SUBSTANCE, ALSO GIVE RISE TO A TISSUE DEBILITATING POISON?

Section No. IV of this Report indicates that *B. tetani* gives rise to tissue-debilitating poisons, for the products of its growth are definitely leucotoxic. In order further to inquire into this problem the following *in vivo* experiment was carried out.

The M.L.D. for rats, of a tetanus toxin obtained from a 10-day growth of bacilli—stored for three months in the ice-chest before use—was first determined. The fraction of the M.L.D. which would invariably give rise to local tetanus but would not cause death was next determined. Mixtures of this quantity of toxin with 2000 million washed but unheated tetanus bacilli (obtained by successive centrifugalisation of rapidly growing cultures suspended in saline) were then made.

(a) Mixture consisting of “local tetanus producing dose” of toxin + Type I bacilli 2000 million.

(b) Mixture consisting of “local tetanus producing dose” of toxin + Type II bacilli 2000 million.

(c) Mixture consisting of “local tetanus producing dose” of toxin + Type III bacilli 2000 million.

(d) Saline + Type I bacilli 2000 million.

Four rats were then inoculated, each with one of the above mixtures. All excepting (d), which remained well, developed local tetanus between the third and sixth days after inoculation; the spasm continued for 7–10 days and thereafter the animals recovered.

This experiment, while failing to prove, or even to suggest, that *B. tetani* does not develop a tissue debilitating substance, shows that a dose of toxin sufficient to cause a temporary disturbance of the anterior horn cells, may not necessarily exhibit, *locally*, qualities sufficiently aggressive to set up infection.

The *spasm-producing* element of the toxin then is not necessarily a determining factor in the mechanism of *infection*.

I have attempted to carry out the converse experiment but have so far met with no success, *i.e.* I have not been able to obtain a culture possessing sufficient aggressive quality, and at the same time of sufficiently

low "spasmin" content, to permit of a differentiation between local debilitating effect with subsequent *infection*, and immediate disturbance due to central nervous system *intoxication*.

(b) AS TISSUE DEBILITATING INFLUENCES CERTAINLY ASSIST IN THE DEVELOPMENT OF TETANUS INFECTION, IS THERE ANY PARTICULAR DEBILITATING INFLUENCE THAT IS OF SPECIAL SIGNIFICANCE IN THIS CONNECTION?

Apart from purely physical considerations such as situation and degree of original trauma, coincident involvement of particular structures, muscle, nerves, vessels, etc., the necrotising influence of the products of certain organisms may play an important part in determining the genesis and evolution of tetanus infection.

That concomitant infection with other organisms might exert such an influence upon the growth of *B. tetani* in wounds, and that this influence might vary with the nature of such concomitant infection, was foreseen by Vaillard and Vincent. The question became insistent when the importance of gas gangrene infections was appreciated in this connection. The following experiments were undertaken with a view to inquiring into the relationship which infection of wounds with *B. Welchii* and *Vibrion septique* might bear to the causation of tetanus.

A preliminary series of experiments was undertaken.

(1) To determine approximately how many washed and heated spores of *B. tetani* could be injected intramuscularly into the guinea-pig without causing the disease.

(2) To determine what dosage of the toxins of *B. Welchii* and *Vibrion septique*, respectively, produced tetanus when injected along with a constant number of spores of *B. tetani*.

In the experiments with *B. Welchii* it was found that 0.2 c.c. of a toxin (which in a dose of 1 c.c. killed one out of three guinea-pigs of 250 grammes weight) sufficed to cause enough local disturbance to determine invariably the onset of tetanus, when injected along with 1000 million spores.

#### A. INFLUENCE OF TOXIN OF *B. WELCHII* ON THE DEVELOPMENT OF TETANUS SPORES *IN VIVO*.

##### *Experiment I.*

Guinea-pig I was inoculated in the right gastrocnemius with 1000 million spores Type I (U.S.A.) in 1 c.c. saline. For the first five days the animal was well; on the sixth and seventh days it limped slightly (? local tetanus); on the eighth day there was no obvious limp, and for the remaining six days during which the animal was under observation it remained well.

Guinea-pig II was inoculated with 1000 million spores Type I plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.). The following day there were signs of local tetanus; on the second day there was definite local tetanus; and on the third day the animal died from generalised tetanus.



Guinea-pig III also received 1000 million spores Type I plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.) in the right gastrocnemius. One hour later 1400 units of *B. Welchii* antitoxin were injected into the left gastrocnemius. The animal showed no signs of tetanus, and remained well during the period in which it was under observation.

### *Experiment II.*

Guinea-pig IA was inoculated in the right gastrocnemius with 1000 million Type II tetanus spores in 1 c.c. saline. It remained well for the first five days; on the sixth there was a slight limp (? local tetanus); on the seventh day there was definite local tetanus; on the eighth general tetanus; then the animal was killed.

Guinea-pig IIA was inoculated with 1000 million Type II tetanus spores, plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.). On the first day the animal appeared to be well; on the second it was moribund, and was then killed.

Guinea-pig IIIA received 1000 million Type II tetanus spores plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.) in the right gastrocnemius. Two hours later 1400 units of *B. Welchii* antitoxin were injected into the left gastrocnemius. For the first five days the animal was well; on the sixth and seventh days it slightly limped (? local tetanus); on the eighth day the limp was less marked; and on the ninth day the animal was well, and it remained well during the period in which it was under observation.

### *Experiment III.*

Guinea-pig IB was inoculated with 1000 million Type III tetanus spores in 1 c.c. saline, in the right gastrocnemius. Guinea-pig IIB received 1000 million spores Type III, plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.). Guinea-pig IIIB was inoculated in the right gastrocnemius with 1000 million spores Type III, plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.), and received 1400 units *B. Welchii* antitoxin in the left gastrocnemius. Guinea-pig IB remained well; Guinea-pig IIB died of tetanus on the third day; and Guinea-pig IIIB, beyond exhibiting slight stiffness of the inoculated limb from the sixth to the fourteenth day after inoculation, remained well.

The deductions to be drawn from these three experiments are self-evident. They indicate that an antitoxin for *B. Welchii* and tetanus antitoxin should be employed together for the routine serum prophylaxis of tetanus. The evidence is unequivocal that the antitoxin of *B. Welchii*, in addition to neutralising its toxin, has the advantage that it completely protects (in the case of the guinea-pig) tissue which has been exposed to the devitalising effect of that toxin against the development of tetanus spores. It is highly probable that the same protecting value would be found in the case of man.

Attention is called to the death of the control animal IA in Experiment II. The death of this animal, while in no way detracting from the validity of the experiments or of the deductions to be drawn from them, indicates that the development of tetanus depends upon a number of different factors. It is not improbable that, in this instance, a certain degree of trauma when the inoculation was made, was responsible for the development of the infection. It is to be specially noted, however, that while the test animal IIA died on the second day after inoculation, the control animal did not die until the eighth day. Moreover the test animal died of acute, almost fulminating tetanus, while the course of the disease in the control animal was much less acute. Experiment II is, therefore, exceptionally instructive: for, while it



shows that the danger of infection with *B. tetani*, and notably the early development of the disease owing to "symbiosis" with *B. Welchii*, could be considerably mitigated—if not completely eliminated—by the use of a combined serum for prophylaxis, it also called attention to the fact, that while such a reagent would probably be of great value, surgical measures should not be less carefully applied because of its introduction.

I call attention to this because, if a combined prophylactic serum were to be introduced, it might lead to the development of a feeling of false security, and too optimistic a view might be taken regarding the probable value of such a combined serum. As a natural sequence its unmerited condemnation would ensue, if the results obtained did not completely bear out expectations.

#### B. INFLUENCE OF THE TOXIN OF *VIBRION SEPTIQUE* ON THE DEVELOPMENT OF TETANUS SPORES *IN VIVO*.

*Vibrion septique* is another of the organisms which are commonly found in wounds, and develops diffusible toxic products. While it is not known whether the toxin of this organism is a particularly active necrotising substance or only has marked oedema-producing properties, it seemed of importance, nevertheless, to determine the possible influence which it might exert upon the development of tetanus.

As the properties of this toxin are at present less well defined than are those of the toxin of *B. Welchii*, the experiments to be described in this section cannot be so clearly interpreted as are Experiments I, II and III, pp. 138–139. This difficulty is enhanced by the fact that, at the time of writing, specific *Vibrion septique* antitoxic serum was not available, so that protection experiments could not be undertaken.

I here wish to record my thanks to Miss Robertson of the Lister Institute of Preventive Medicine for placing at my disposal a quantity of the toxin of *Vibrion septique*.

The methods employed and the results obtained in making this investigation were as follows:

##### *Experiment IV.*

Guinea-pig A was inoculated in the right gastrocnemius with 0.25 c.c. *Vibrion septique* toxin plus 0.75 c.c. saline. On the first day there was slight oedema and stiffness of the leg. On the second day the oedema was palpable, but not extensive; there was still stiffness of leg. On the third day the oedema was almost gone and the leg less stiff. On the fourth day the animal had almost recovered, and on the fifth day it was well, and remained so during the period of the experiment.

Guinea-pig B was inoculated with 0.25 c.c. *Vibrion septique* toxin, plus 0.25 c.c. saline plus 1000 million tetanus spores Type I. On the first day there was slight oedema and stiffness of leg. On the second day oedema palpable but not extensive; and there was still stiffness of leg. On the third day the animal was recovering, and on the fourth it had almost recovered. On the fifth day it was well, and remained so.

Although the oedema resulting from the injection of 0.25 c.c. of this toxin was quite as marked as, if not more marked than, that produced by 0.2 c.c. of the toxin of *B. Welchii*, it is remarkable that tetanus did not develop. I therefore decided to make the test much more stringent, employing 1 c.c. of the toxin plus 1000 million tetanus spores. The result of an experiment employing this technique is shown below.

### *Experiment V.*

Animal C was inoculated in the right gastrocnemius with 1 c.c. *Vibrion septique* toxin plus 0.5 c.c. saline. On the first day there was marked oedema over the whole of the injected limb; on the second the oedema was more marked and extending over the abdominal wall, soft on palpation. The next day the oedema was reduced and the animal lively; limb stiff. On the fourth day the oedema was disappearing; leg still stiff. On the fifth day the animal had recovered, and remained well till the conclusion of the experiment.

Animal D received 1 c.c. *Vibrion septique* toxin plus 1000 million Type I tetanus spores. On the first day there was marked oedema over the whole of the injected limb; on the second, oedema as in Animal C. On the third day the oedema was reduced and the animal lively, but limb stiff. Fourth day, oedema almost gone; leg still stiff. On the fifth day the animal was well, and remained so.

A slight stiffness of the limb remained in both animals until the completion of the experiment, which was considered to have occurred on the fourteenth day. The animals have remained well, and the stiffness is slowly disappearing. This experiment is striking, in that, although the oedema produced in the animals was much more marked than that resulting from the injection of 0.2 c.c. of the toxin of *B. Welchii* employed in the previous series of experiments, no development of tetanus occurred.

This result was scarcely expected, and the experiment was therefore repeated.

### *Experiment VI.*

Animal E was inoculated with 1000 million Type I tetanus spores suspended in 1.5 c.c. of saline. Animal F with 1 c.c. of *Vibrion septique* toxin plus 1000 million Type I tetanus spores. These animals both remained well, so corroborating the findings indicated in Experiment V.

As the disturbance produced by *Vibrion septique* toxin—oedema production—was so pronounced, it seemed remarkable that tetanus did not develop. The experiment was repeated a second time, using spores of a representative Type III tetanus batillus. The findings of this experiment are as follows:

### *Experiment VII.*

Animal G was inoculated in the right gastrocnemius with 1000 million Type III tetanus spores suspended in 1.5 c.c. saline. It remained well. Animal H was inoculated with 1000 million Type III tetanus spores plus 1 c.c. *Vibrion septique* (volume 1.5 c.c.). There was marked oedema on the first day, but the animal was active. On the second day there was general tetanus, and the animal was killed.

As specific antitoxin for *Vibrion septique* was not available, it was decided not to complete the present series of experiments using Type II spores: for the results shown in Experiment VII indicate that *Vibrion septique* may, like *B. Welchii*, play an ancillary part in the causation of tetanus.



The negative results obtained on three occasions when Type I spores were used indicate, however, that the toxin of *Vibrion septique* is probably a less constant factor in stimulating the growth of *B. tetani* in the tissues, than is that of *B. Welchii*. In view, however, of its frequent occurrence, and in view of the fact that it sporulates more readily than does *B. Welchii*, its capacity for doing harm is possibly extended over a more prolonged period than is the case with *B. Welchii*. It would seem advisable therefore, that antibodies to the toxin of *Vibrion septique* should also be included in serum used for the prophylaxis of tetanus.

CONCLUSIONS FROM THIS SERIES OF EXPERIMENTS, pp. 138-142.

1. There is good ground for believing that the ancillary part played by *B. Welchii* in the causation of tetanus is clearly defined. The capacity of this organism for doing harm in the connection under consideration can be almost eliminated by the use of the antitoxin for *B. Welchii*.

2. The capacity of the toxin of *Vibrion septique* for stimulating the growth of tetanus spores *in vivo* is more variable than is that of *B. Welchii*. Experiment VII indicates, however, that it too may play a part in the causation of tetanus.

3. It follows from conclusions 1 and 2 that antibodies to the toxins of *B. tetani*, *B. Welchii*, and *Vibrion septique* should be included in all serum employed for the prophylaxis of tetanus.

4. While such a polyvalent serum promises to reduce still further the incidence of tetanus, we may not assume that it would absolutely eliminate that disease, for infections other than those dealt with in this communication may also play a part in stimulating the growth of *B. tetani* in wounds.

(c) AS CONCOMITANT INFECTION WITH CERTAIN ORGANISMS STIMULATES THE GROWTH OF SPORES OF *B. TETANI* IN TISSUE, IS IT NOT POSSIBLE THAT THE CONVERSE MAY BE EQUALLY TRUE? AT LEAST, MAY NOT SOME CONCOMITANT INFECTIONS REDUCE THE TOXOGENIC CAPACITY OF *B. TETANI* UNDER CERTAIN CIRCUMSTANCES WHICH ARE AT PRESENT UNKNOWN?

The facts dealt with in this Sub-section of the Report were obtained, not in making inquiry into the above question, but in conducting an investigation with another object in view. This problem is complementary to (b), p. 138. In examining wound exudates from men showing no evidence of tetanus, Miss Cayley obtained three consecutive cultures, which failed to develop toxin when tested by the ordinary methods, but nevertheless contained bacilli in large numbers having the morphological characters of *B. tetani* and, on investigation by the method described in a previous com-



(c) that the conditions under which the organisms were growing rendered them temporarily non-toxogenic, or that toxin was destroyed as rapidly as it was produced.

The three cultures under consideration will be hereinafter designated C 5, C 14 and C 15.

(i) C5 on the 7th day after seeding showed the presence of organisms of the tetanus morphological type, the sporogenes type and oval end-sporing bacilli.

(iii) C 15 showed tetanus type on the 9th day of incubation, sporogenes type also being present.

• B. Sub-cultures were made from these original meat broth tubes in the enriching medium described in a previous communication (*Journal R.A.M.C.*, Dec. 1917).

(i) C5 in the enriching medium gave after three days' incubation a strong growth of bacteria having the morphological characters of *B. tetani*.

(ii) C 14 behaved in a similar way, but sporulation was not marked until the 4th day of incubation.

(iii) C 15 behaved exactly as did C 14.

C. These cultures were then centrifugalised, washed in saline, and suspensions made and standardised by the opacity method, to contain 2000 million bacilli per c.c. The suspensions were then heated and phenolated, following the standard technique, and finally exposed to the action of Types I, II, and III, agglutinating sera. The results obtained are shown in Table XV.

Table XV.

[illegible]

Absorption of agglutinin tests were then carried out again using the standard technique, and results shown in Table XVI were obtained.

Table XVI A.

*Absorption of Type I Serum by Cultures C 5, C 14, C 15.*

Culture	Unabsorbed serum				Absorbed serum							
	Agglutination				Homologous bacillus added				Test bacillus added			
	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800
	C 5	+	+	+	-	+	-	-	-	-	-	-
C 14	+	+	+	-	+	-	-	-	-	-	-	-
C 15	+	+	+	-	+	-	-	-	-	-	-	-
Control	+	+	+	-	-	-	-	-	-	-	-	-

Table XVI B.

*Absorption of Type II Serum by Cultures C 5, C 14, C 15.*

Culture	Unabsorbed serum				Absorbed serum							
	Agglutination				Homologous bacillus added				Test bacillus added			
	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800
	C 5	-	-	-	-	+	+	+	+	-	-	-
C 14	-	-	-	-	-	+	+	+	+	-	-	-
C 15	-	-	-	-	-	+	+	+	+	-	-	-
Control	+	+	+	+	-	-	-	-	-	-	-	-

Table XVI C.

*Absorption of Type III Serum by Cultures C 5, C 14, C 15.*

Culture	Unabsorbed serum				Absorbed serum							
	Agglutination				Homologous bacillus added				Test bacillus added			
	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800
	C 5	-	-	-	-	+	+	+	+	-	-	-
C 14	-	-	-	-	-	+	+	+	+	-	-	-
C 15	-	-	-	-	-	+	+	+	+	-	-	-
Control	+	+	+	+	-	-	-	-	-	-	-	-

The findings shown in Tables XV and XVI indicate that the three cultures under consideration were Type I tetanus bacilli, and, in view of their failure to produce toxin when tested in the ordinary way, inquiry had to be made to determine whether :

- (a) They were intrinsically atoxic.
- (b) The circumstances of their growth reduced their toxogenic capacity.

D. Animal experiments were therefore undertaken to investigate these points.

(1) In the first place sub-cultures into ordinary peptone broth were made from the original meat cultures, and were incubated anaërobically for six days.

Microscopical examination of the growths was then made to determine the

presence of organisms bearing spherical terminal spores. A massive inoculation—0.5 c.c.—of these growths was made intramuscularly into each of three rats. All the animals remained well, so confirming the original experiment. The experiment was repeated with mice and the same result was obtained.

(2) As it seemed possible that the tetanus bacilli might have been introduced into the enriching medium through technical error, sub-cultures were made in broth from the tubes containing growths of C 5, C 14 and C 15 in the enriching medium. After 10 days 0.5 c.c. of each of these sub-cultures was inoculated into mice.

Notwithstanding these massive inoculations two of the mice remained well. That inoculated with C 14 died from tetanus on the 5th day. It seemed improbable then, that the tetanus bacilli had been introduced into the enriching medium during any of the various processes of its preparation. In order further to ensure that the medium was not a source of error, sub-cultures from the original meat tubes were made in three different batches of enriching medium, and agglutination tests were repeated with the cultures so obtained. All gave consistent results.

*Note.* Before any batch of enriching medium is used in the laboratory, it is examined by cultural methods to ensure sterility, and where animal experiments are to be performed with growths of organisms in this medium, it is inoculated into mice to ascertain that the medium itself does not exhibit toxic properties.

(3) The following experiments were then set up to determine, if possible, whether the tetanus bacilli in C 5, C 14 and C 15 were themselves atoxic, or whether their toxogenic capacity was interfered with by concomitant growth of other bacteria present in the cultures.

(i) Four tubes of meat broth, *a*, *b*, *c*, and *d*, were each inoculated with an equal quantity of the same culture of Type I bacilli—the U.S.A. standard culture which was known to be toxic.

Into Tube “b”	was then seeded	some of the original meat culture	of C 5,
”	“c”	”	”
”	“d”	”	”
			C 14,
			C 15.

Tube “a,” which was inoculated with the standard U.S.A. culture only, served as a control.

All four tubes were incubated for eight days, and then 0.25 c.c. of each culture—again a massive inoculation for mice—was injected subcutaneously into mice.

The mouse inoculated with “a” died from tetanus within 24 hours: that inoculated with “b” showed tetanic symptoms from the 3rd to the 8th day, but eventually recovered; that inoculated with “c” remained well; while the animal which received “d” developed tetanus only on the 5th day and died from the disease on the 6th.

(ii) The experiment was repeated but the tubes in this instance were incubated for 10 days.



The animal inoculated with "a" died within 24 hours; that with "b" showed tetanic symptoms from which the animal ultimately recovered—the onset of the condition occurring six days after inoculation. The animals inoculated with "c" and "d" remained well.

(iii) Exactly similar experiments were then carried out using cultures from mixed *inocula* of Type II and Type III bacilli and C 5, C 14 and C 15. The cultures were incubated for eight days and inoculated as in (i) and (ii). In neither case, however, did any of the growths prove atoxic.

(iv) Experiment (i) was repeated a second time and gave unequivocal confirmation of the previous findings.

(v) As experiments (i) and (ii) of this Sub-section indicated that the toxic qualities of mixed cultures might vary from day to day I made four sub-cultures each of C 5, C 14 and C 15 for inoculation on different days.

One series of tests was made after four, one after six, one after eight, and one after ten days' incubation. The *inocula* consisted of 0.5 c.c. in each instance, and the animals used were rats. All the animals in this series of experiments survived, and none showed evidence of tetanus.

(vi) The experiments described in (i), (ii) and (iv) indicate that co-incident growth of other organisms may prevent the development of tetanus toxin in cultures.

In order finally to dispose of the question, sub-cultures from the original meat tubes of C 5, C 14 and C 15, were made in the enriching medium, and the growths so obtained were centrifugalised, washed, and heated, as for the preparation of suspensions for agglutination. In doing this, special precautions were taken to prevent the occurrence of contamination. A specimen of each of the suspensions so obtained was retained for animal inoculation, and the bulk of each suspension was agglutinated. Again all three cultures reacted well in the presence of Type I agglutinating serum.

The specimens removed from each suspension before agglutination were dealt with thus:

(a) 0.1 c.c. of suspension of C 5 was mixed with 1/4 M.L.D.—for mice—of *Welchii* toxin. The mixture, so prepared—total volume 0.2 c.c.—was injected subcutaneously into a mouse. The animal developed tetanus and died on the third day.

(b) 0.1 c.c. of suspension of C 14 + the same quantity of *Welchii* toxin was injected into a second mouse. The animal developed tetanus, and was killed on the second day after inoculation.

(c) An exactly similar experiment in which the suspension of C 15 was tested resulted in the development of tetanus on the third day after inoculation.

(d) A control mouse, inoculated with the *Welchii* toxin alone, remained well.

The experiment was repeated using a smaller dose of *Welchii* toxin, and substantially the same result was obtained.

Commenting on these results, it is remarkable that there is such definite evidence of loss of toxogenic power, when the Type I culture is grown in a mixture along with the cultures under consideration. It is also remarkable that a similar result was not obtained with the Type II and Type III bacilli—Experiment (iii), p. 146.

This finding might suggest that cultures of Type I bacilli were more susceptible to depression of their toxogenicity by certain mixtures than were cultures of Type II and III. This is possibly so; but, short of carrying out a long series of quantitative experiments with numerous strains of each Type, such a deduction is not permissible. It is more probable that the particular strain of Type I used was—perhaps only temporarily—less toxogenic than the strains of Types II and III employed, and therefore the conditions necessary for demonstrating depression of their toxogenicity were not realised.

The important point is, as the experiments definitely show, that certain mixtures of organisms depress to a greater or less extent the toxin-producing capacity of certain strains of tetanus bacilli when grown together with them. Experiment (vi), p. 142, of the series shows that spores of tetanus bacilli obtained from such non-toxic mixtures are definitely toxogenic when inoculated into animals under suitable conditions.

(d) PRELIMINARY EXPERIMENTS CONDUCTED WITH A VIEW TO THE  
ELABORATION OF A METHOD FOR STUDYING INFECTION  
WITH *B. TETANI*.

*Investigations conducted for the purpose of determining a suitable tissue debilitant, which might be employed for starting the infective process in various animals.*

The experiments already described indicate that a method might be elaborated for studying the various problems of the prophylaxis and therapeutics of tetanus from the standpoint of *infection* instead of from the standpoint of *intoxication*.

Could this be done, a more rational view of the pathogeny of the natural disease would be obtained, and, it is not improbable, that improvements in the methods of prevention, if not in the methods of treatment, might be suggested by the findings obtained.

The primary object of the investigation was to obtain, if possible, a standard method of *infection*. This would constitute a basis for the study of the prophylactic values of *anti-bacterial* and *antitoxic* sera; and also for the study of the action of “mono-typical” sera in order to determine whether they are or are not specific.

The experiments described on p. 142 show that the toxin of *B. Welchii* might be used for this purpose in guinea-pigs and in mice; it also sets up infection in the rat. The employment, however, of a biological reagent of this nature, for the purpose in view, presents considerable difficulties, owing to its instability, and owing also to its somewhat inconstant



effect upon different members of even one animal species, because of the variation in susceptibility exhibited. I have employed it in conducting a series of observations which will be described later, but found it unsatisfactory; because, if reliable results are to be obtained, it is essential that a complete series of experiments be performed at one time. This precluded its use in experiments in which guinea-pigs were employed, owing to the expense incurred, and, as will be seen from subsequent observations, infection experiments are difficult to carry out under standard conditions.

When a standard number of spores together with the debilitant are injected into animals, it is found that a narrow margin exists between the degrees of tissue debility which, on the one hand, will certainly give rise to the development of tetanus and which, on the other hand, will cause death in most animals even though protected by large doses of antitoxin.

By the term "a large dose of antitoxin," I mean such a (relative) quantity as would be quite impracticable as a prophylactic dosage in man; *e.g.* 50 units for a guinea-pig is equal to 12,000 units for man.

A number of substances were therefore investigated in an attempt to obtain a more stable, and therefore more constant irritant than *Welchii* toxin, or any other biological product, and which could moreover be standardised by physical or chemical methods. The substances examined were:

1. *Lactic Acid*. Various solutions of this reagent were tried both on guinea-pigs and mice, which were inoculated at the same time with a standard number of spores. The results were so inconstant that the reagent was obviously unsuitable for the purpose in view.

2. *Trimethylamine*. This reagent was also tried and was found to give fairly constant results in the case of guinea-pigs; but its marked alkalinity and its tendency to undergo decomposition proved it to be a less reliable substance than saponin for the purpose of setting up infection in these animals.

Trimethylamine could not be satisfactorily used in the case of mice, for a somewhat unexpected result was obtained with them, illustrating the difficulty of the problem under consideration, and which I think is worthy of record.

Twelve mice were inoculated subcutaneously with the following mixtures of spores and trimethylamine.

(a)	100 million	Type I	spores	+ 0.1 c.c.	trimethylamine	33 %	+	-
(b)	"	"	II	"	"	"	+	-
(c)	"	"	III	"	"	"	+	-
(a')	"	"	I	+ 0.05 c.c.	"	"	+ 0.05	saline
(b')	"	"	II	"	"	"	+	"
(c')	"	"	III	"	"	"	+	"
(a'')	"	"	I	+ 0.025 c.c.	"	"	+ 0.075	saline
(b'')	"	"	II	"	"	"	+	"
(c'')	"	"	III	"	"	"	+	"
(a''')	"	"	I	+ 0.0125 c.c.	"	"	+ 0.0875	saline
(b''')	"	"	II	"	"	"	+	"
(c''')	"	"	III	"	"	"	+	"

The volume of each *inoculum* was 0.3 c.c.



(1) Animals (*a*), (*b*), and (*c*), were all found dead the morning following the inoculation. They showed no evidence of tetanus.

(2) (*a'*), (*b'*), and (*c'*), each developed an eschar about  $\frac{1}{4}$  of an inch in diameter at the site of injection, two days after inoculation. Notwithstanding this, they remained well, were lively, took food greedily, and after a further lapse of three or four days, all but one had recovered. The animal which died—(*b'*)—did not show any evidence of tetanus, and only succumbed after the eschar had completely healed.

(3) (*a''*) developed definite tetanus on the 7th day after inoculation and was killed.

(*b''*) showed demonstrable tetanic spasm on the 3rd day and it, too, was killed.

(*c''*) died from tetanus on the 2nd day.

(4) (*a'''*), (*b'''*), and (*c'''*), all remained well.

From the above experiments it is seen that only the third series of tests gives results which are at all encouraging; but the time variation before the onset of the disease, makes the employment of diluted trimethylamine impracticable for experiments designed to investigate the prophylactic value of sera.

It is very remarkable that those animals which received 0.05 c.c. of the reagent, although they showed marked local disturbance, did not succumb to tetanus; while those which received 0.025 succumbed although they showed practically no local disturbance.

3. *Saponin*. The use of this reagent was suggested to me by Dr F. Ransom and I wish to thank him cordially for his advice.

The following experiments were carried out to determine how this substance might be employed in order to set up infection. It is to be noted, that the great advantage of saponin lies in the fact that its solutions can be standardised by *physical* methods, and it can be sterilised by autoclaving, without losing its irritant properties; solutions should not, however, be autoclaved more than once.

Five guinea-pigs were inoculated intramuscularly with the following mixtures:

Animal 1,	saponin	1/100	0.2 c.c.,	saline	0.2 c.c.,	Type I spores	200 million—	Vol. =	0.6 c.c.
„ 2,	„	1/200	„	„	„	200	„	„	„
„ 3,	„	1/400	„	„	„	200	„	„	„
„ 4,	„	1/800	„	„	„	400	„	„	„
„ 5,	„	1/1600	„	„	„	400	„	„	„

*Number 1* showed evidence of local tetanus on the morning of the 2nd day, rapidly developed tetanic convulsions and was therefore killed.

*Number 2* had stiffening of the inoculated limb on the evening of the 2nd day and was found suffering from generalised tetanus on the morning of the 3rd day. It was then killed, as the possibility of recovery was remote.

*Numbers 3, 4 and 5* remained perfectly well. This experiment gave an indication of how a standard infection might be set up in the guinea-pig—

at least so far as a standard infection of any kind can be set up in any animal. Subsequent experiment showed that 200 million spores + 0.2 c.c. of 1/200 saponin gave consistent results, tetanus invariably developing on the 2nd or 3rd day after the intramuscular injection of the mixture. Equal volumes of 1/300 dilution of the same sample of saponin in presence of the same number of spores did not give constant results.

A similar series of tests was made with mice, dilutions of saponin 1/50-1/400 being tested, but the results obtained were entirely negative. The test was done in triplicate, using spores of all three types of *B. tetani*. The animals remained well when the lower concentrations were used, and showed absolutely no evidence of tetanus, but succumbed to saponin intoxication, usually within 24 hours, when the higher concentrations were injected.

The results of these experiments, dealing with the employment of irritants for setting up infection, show the following interesting points.

(i) *Welchii* toxin, although unsatisfactory for performing quantitative tests, proved to be the most consistent of the reagents investigated, in respect of its power of producing infection in a variety of animal species.

(ii) The chemical reagents are less consistent, *e.g.* saponin sets up infection in the guinea-pig but not in the mouse.

(iii) The dilution of the reagent employed may have a very marked influence, and sometimes an unexpected one, in respect of its power to give rise to infection in different animals. This fact is strikingly illustrated in the case of trimethylamine. When this reagent is used on mice it shows, that not only the degree, but also the nature of the tissue-debilitating lesion may profoundly affect the development of tetanus.

(iv) In the guinea-pig, saponin can be used to set up an infection, and gives fairly constant results. The animals die two to three days after inoculation. A method of testing the *anti-infective* properties of sera in guinea-pigs seems, therefore, to have been obtained.

(v) The experiment, in which the infection-stimulating properties of saponin were investigated, shows that the degree of tissue destruction, rather than the number of spores inoculated, is the determining factor in causing the development of *infection*. Thus animals 4 and 5 did not develop the disease although they received twice the number of spores injected into animals 1, 2 and 3.

#### (e) EXPERIMENTS DEALING WITH PROPHYLAXIS WITH ANTI-TETANIC SERUM.

(i) *Degree of tissue destruction in its relation to the causation of tetanus.*

(ii) *Degree of immunity conferred by varying the dose of antitoxin.*

In the previous Sub-section, pp. 147-150, attention is called to the important bearing which the degree of tissue debility may have upon the



development of tetanus, when a constant number of spores are inoculated. This raises the question: "Does antitoxin, used in doses having a ratio practicable in man, prevent a fatal issue in experimental animals, when the degree of tissue destruction is great?"

In an attempt to inquire into this problem the following experiment was carried out:

Four rats, (*a*), (*b*), (*c*) and (*d*), each weighing approximately 100 grammes, received 40 units of antitoxin; the antitoxin used was mark "B.W. horse 13."

After an interval of two days animal

( <i>a</i> )	was inoculated with a mixture of <i>Welchii</i> toxin + 500 million Type I spores,
( <i>b</i> )	" " " " + 500 " " II "
( <i>c</i> )	" " " " + 500 " " III "
( <i>d</i> )	" " " " + saline.

The dose of *Welchii* toxin chosen was that which produced a marked oedema of the whole limb when injected by the intramuscular route. Controls which had received no antitoxin were also injected with the same mixtures. The control animals and also animals (*a*), (*b*) and (*c*) all developed tetanus and died, the fatal issue in the experimental animals as compared with the control animals being delayed only for 24 to 48 hours. Animals injected with tetanus spores only remained well. Animal (*d*)—*Welchii* toxin alone—naturally developed a marked oedema of the inoculated limb, but recovered completely in four or five days.

The experiment was repeated on mice, which were given 5 units of antitoxin two days before the inoculation of spores and *Welchii* toxin. An exactly similar result was obtained.

If the dose of antitoxin used in these experiments be expressed as gramme weight equivalents for man the ratio in each instance is:

40 units to a rat of 100 grammes is equal to
24,000 " " man of 60 kilos.
5 " " mouse of 15 grammes is equal to
20,000 " " man of 60 kilos.

The results indicate that the development of infection with *B. tetani*, in passively immunised animals, depends largely upon *quantitative* factors, one of which is, the degree of tissue destruction in the infected area. While, therefore, we may improve upon the methods of serum prophylaxis at present in use, we cannot hope to eliminate the disease completely by the prophylactic use of antitoxin.

Another most important point is suggested by the experiment, viz. that a factor of special import in the prophylaxis of the disease, is early and free removal of devitalised tissue. *Surgery is as important as serum prophylaxis in the prevention of tetanus.*



This finding prompted a further query: "Will a large dose of antitoxin give more adequate protection than a smaller one against a fatal result from infection with *B. tetani* under standard conditions—so far as any infection can be standardised?"

This subject is of great importance and can only be investigated by experiments based on *infection*, as opposed to experiments based on *intoxication*. The point at issue is not how long the passive immunity conferred by a dose of antitoxin lasts, but the degree of immunity conferred by antitoxin during the period between the 3rd and 6th day after the reception of the injury.

It has been my experience in conducting infection experiments in guinea-pigs, that if the animal lives for six days after it has been inoculated without showing evidence of tetanus, the disease will probably not develop at all; or, if it does, the infection, in a considerable number of instances, will only result in local tetanus and subsequent recovery. Stated in terms of the natural disease in man, the question really becomes—*Can we hope to prevent the occurrence of a percentage of fulminating cases of the disease by the routine employment of a larger initial prophylactic dose of antitoxin than that which is at present in use?*

In the earlier series of infection experiments in which guinea-pigs were used, the serum was injected two or three days prior to the inoculation of the infecting material—mixture of spores and irritant—and the following results were obtained:

Table XVII.

No. of animals	Ratio of anti-toxin for use	Units	Recovered	Died
21	1,450	6 or less	0	21
2	2,900	12 units	0	2
6	6,000	25 „	1	5
6	12,000	50 „	2	4

In order further to investigate this problem I performed the following experiment:

Two sets of 3 guinea-pigs each, were immunised with various doses of antitoxin; Lister Institute, No. 136 A serum was used. Two hours before the serum was injected, all the animals were inoculated intramuscularly with a mixture of 0.2 c.c. 1/200 saponin solution + 200 million spores, the total volume of the *inoculum* being 0.6 c.c. Table XVIII, p. 153, shows the details and results of the experiment.

Commenting on these results it is seen, that with the five units of antitoxin the onset of the disease was not delayed so markedly as when larger quantities were employed. With 10 units of antitoxin the late onset of the disease and its relatively slow progress indicate that a larger initial prophylactic dose of antitoxin will probably be of considerable value, both in limiting the incidence of the disease and in beneficially altering its clinical characters, by rendering the latter more chronic and so allowing of more adequate therapeutics.

Table XVIII.

Animal	Saponin	Spores	Anti-toxin	Ratio	Result
A	1/200 0.2 c.c.	200 million Type I	5	1200	Local tetanus 2nd day, generalised 4th day
B	"	" " I	10	2400	Remained well till 13th day, developed local tetanus—generalised three days later
C	"	" " I	20	4800	do. do.
A'	"	" " II	5	1200	Local tetanus 3rd day, definitely generalised 6th day
B'	"	" " II	10	2400	Remained well till 13th day, developed local tetanus—generalised three days later
C'	"	" " II	20	4800	Died 4th day. No evidence of tetanus

The experiments are too few in number, and the conditions under which they were carried out too variable—I refer, particularly, to the experiments summarised in Table XVII, p. 152—to permit of any far-reaching conclusions being drawn from the results obtained; they are, nevertheless, extremely suggestive.

I would here call attention to an apparent discrepancy between the protective values of the sera as shown in the results indicated in Table XVII, p. 152 and Table XVIII, p. 153.

In the experiments shown in Table XVIII the serum appears to exert more marked protective influence than in the experiment shown in Table XVII. This may depend upon the fact, that the serum and the infecting material were inoculated on different days in the first instance, and on the same day in the second instance.

The obvious criticism which might be made of these experiments is, that the ratios of antitoxin used are so high, that the figures are of no import. It must be clearly appreciated, however, that the conditions which determine infection with *B. tetani* in the guinea-pig, are almost certainly very different from those which determine infection in man. One of the most striking features of the infection experiments which I have performed, is the marked degree of tissue debility that must be induced to ensure infection in these animals. It is well known that in unprotected men, on the contrary, a comparatively small lesion may suffice to set up a fatal infection with *B. tetani*.

Further, it is very questionable what interpretations we should put upon the ratios expressed in these Tables, for clearly, a dose of 500 units, for example, will only be neutralised by ten times the quantity of toxin required to neutralise a dose of 50 units. But man is certainly more susceptible to tetanus toxin (spasmin)—probably *much* more susceptible—than the guinea-pig; and there is some ground for believing that he is also much more susceptible to infection. Therefore the ratios stated in Tables XVII and XVIII may not be so fantastic as they appear to be.



Moreover, the extent of the exposed surfaces in large wounds in men, probably tends to make a gramme weight basis of comparison between man and guinea-pig a more correct index of the relative conditions obtaining in the two species, than would a simple "dose for dose" comparison.

It is these factors which make protection experiments in guinea-pigs, when tested by the infection method, extremely difficult to interpret. For with the excessive tissue destruction which is required to ensure infection in that animal, the tetanus bacilli develop in a situation essentially *outside* the body. By reason of their development in this situation, they are protected from the body-fluids, and probably grow with extreme rapidity. In a certain number of cases of the natural disease as it occurs in man, when the tissue lesion is less extensive, the contact between the living tissue and the organism may be fairly intimate. In such circumstances reaction to infection may play a not unimportant part in preventing the occurrence of the disease.

Another point which must not be lost sight of, is, that the concentration of spores in the circumscribed, though relatively large, devitalised area, in experimental infection, is much greater than is likely to occur in most cases of natural infection. This will naturally tend to make the experimental disease exhibit a fulminating character; but unfortunately, a large number of spores—200 million were used throughout the series of tests—appears to be necessary to ensure infection. With small numbers one cannot be certain of introducing a sufficiency of viable spores, to cause death of control unprotected animals within three days from inoculation.

The setting of a time limit of three days for the controls is, of course, arbitrary; but, unless a short period be taken for this purpose, the experimental error assumes serious proportions, owing to the number of survivals which occur, even in the control animals, when the period between inoculation and onset of the disease is lengthened.

#### CONCLUSIONS.

1. As one would naturally expect, the extent and degree of tissue devitalisation are an extremely important factor in determining the occurrence and termination of infection with *B. tetani*.

2. If the extent and degree of devitalisation exceed certain limits, it is certain that in animals—therefore also probably in man—no amount of antitoxin within practical limits, will give complete protection from the disease.

3. There is some evidence that the administration of a large initial prophylactic dose of antitoxin may give adequate protection against tetanus to a larger number of men than a small dose. What I mean is, that while a prophylactic dose of, for example, 500 units, will prevent the occurrence of a certain high percentage of tetanus cases among men, an increased dose to 1000 units would not result in the prevention of double the number of cases.



It would, however, probably increase the percentage of successful applications of serum prophylaxis, although the increase in percentage may be only small.

(f) INVESTIGATION OF ANTI-INFECTIVE PROPERTIES OF ANTITOXIC AND ANTI-BACTERIAL SERA.

Experiments undertaken to determine

(i) *Whether antitoxic sera exhibit anti-infective qualities in relation to the serological Type of the infecting bacilli.*

(ii) *Whether improvement in serum prophylaxis may be looked for from the employment of sera possessing anti-bacterial as well as anti-toxic properties.*

The experiments carried out *in vitro* which were discussed in the previous Section of the Report, pp. 120–135, show that *antitoxic* sera neither exhibit specific neutralising properties to the spasm-producing toxins of the three Types, nor do they stimulate phagocytosis of *B. tetani*. The experiments did suggest however, that antitoxic serum might, on the contrary, specifically neutralise the leucotoxic quality possessed by certain specimens of toxin.

It seemed possible, though improbable, that *mono-typical antitoxic* sera might, under certain circumstances, exhibit *anti-infective* properties specific to the Type of the infecting organism.

Experiments were undertaken with a view to investigating this point.

A. INFECTION EXPERIMENTS IN WHICH *WELCHII* TOXIN WAS USED AS THE TISSUE DEBILITANT.

*Experiment VIII.*

Six guinea-pigs, 1, 2, 3, 4, 5 and 6, were used; animals 1, 2 and 3 each received 2 c.c. normal rabbit serum while 4, 5 and 6 were passively immunised with 2 c.c.—50 units—of the serum of a rabbit prepared with Type II toxin.

On the second day after the administration of the serum, 200 millions of spores were injected by the intramuscular route, together with *Welchii* toxin. The dose of *Welchii* toxin in each instance was 0.1 c.c. = 1/2 the mouse M.L.D. of the particular toxin employed. The details of the experiment and the results obtained are shown in the following Table.

Animal No.	Spores 200 million, <i>Welchii</i> toxin 0.1 c.c., volume = 0.6 c.c.	Serum used, volume 2 c.c., antitoxin 50 units	Result
1	Type I	Normal rabbit	Died from tetanus 2nd day after inoculation
2	„ II	„	do. do.
3	„ III	„	do. do.
4	„ I	Type II antitoxin	Generalised tetanus 3rd day
5	„ II	„ „	Remained well
6	„ III	„ „	Generalised tetanus 3rd day

## Experiment IX.

The result of Experiment VIII was encouraging, and it was therefore decided to repeat the test with mice. In this instance, the *antitoxic* sera were mixed with the spores and the *Welchii* toxin, and all three reagents were injected together. The following are the details of the experiment.

*Inocula* (a) spores = 100 million.

„ (b) antitoxin = 0.6 units.

„ (c) *Welchii* toxin = 1/2 mouse M.L.D.

Antitoxin Type I = Horse 13 B.W.

„ Type II = Rabbit I'.

Inoculation of spores, antitoxin, and tissue debilitant, made together.

Animal No.	Spores Type	Antitoxin Type	<i>Welchii</i> toxin	Result
1	I	Nil—saline only	1/2 M.L.D.	Dead 2nd day
2	II	„ „	„	„
3	III	„ „	„	„
4	I	Type I—0.6 units	„	Remained well
5	II	„ „	„	Generalised tetanus 4th day
6	III	„ „	„	„ „ 5th „
7	I	Type II—0.6 units	„	General tetanus 2nd day
8	II	„ „	„	Remained well
9	III	„ „	„	Generalised tetanus 3rd day
10	Nil	Nil	„	Ill for one day but recovered

*Note.* In the above experiment, the Type I antitoxin was obtained from Dr O'Brien and the vehicle of the antitoxin was therefore horse serum. It is not strictly comparable therefore with the Type II antitoxin which was prepared by myself—the vehicle of the antitoxin being rabbit serum. I here wish to record my thanks to Dr O'Brien for placing this serum at my disposal, as my Type I rabbits were not available for use at that time.

The above results are very striking, but the experiments might justifiably be criticised on the following grounds:

1. It may be purely a coincidence that the *homologous* animals survived. Admittedly the chance of such occurring is remote; but, in view of the complexity of the experiments, it cannot be excluded.

2. Using the toxin of *B. Welchii*—a biological reagent—as the tissue debilitant, introduces a potential error, for it is probable that not every individual of a species is equally susceptible to this toxin. Many attempts have been made to repeat the experiment, but using chemical agents to light up the infection. None of these, however, proved successful; for, either the controls did not take the disease, or the amount of tissue debilitant that had to be introduced to ensure infection, resulted in the death of a number of the animals, not from tetanus, but from poisoning with the chemical agent employed.

3. The amount of *Welchii* toxin used—1/2 M.L.D.—appears to be excessive; but again, it is necessary if infection is to occur regularly. Such a large dose of tissue debilitant leaves but a slight margin to allow of accidents. In an attempt to overcome certain of these criticisms the experiment was repeated on guinea-pigs.

This test was performed one week after the completion of Experiments VIII and IX; the same specimen of *Welchii* toxin was employed, in the same dose, *by volume*, as before. When this experiment was carried out, I did not fully appreciate the rapidity with which *Welchii* toxin deteriorates. As the results of Experiment X show, this deterioration of toxicity really made the test invalid.

I quote the experiment to illustrate one of the many difficulties which are encountered in conducting infection experiments with *B. tetani*, and to emphasise how very difficult it may be to interpret the results obtained.

### *Experiment X.*

The details of this experiment are shown as follows.

- (a) Animals = guinea-pigs.
- (b) Spores dose = 250 million.
- (c) *Welchii* toxin = same dose, and same volume of toxin as used in Experiment VIII.
- (d) Antitoxin Type I = Horse 13 B.W.
- (e) „ „ II = Rabbit I'.
- (f) Dose of antitoxin = 5 units.
- (g) Method of inoculation = Mixtures made of spores, *Welchii* toxin and tetanus antitoxin, incubated for 30 minutes at 37° C. and injected simultaneously by the intramuscular route.

Animal No.	Spores Type	<i>Welchii</i> toxin	Antitoxin 5 units		Result
1	I	„	Nil—saline only		Local tetanus 9th day, generalised 14th day
2	II	„	„	„	General tetanus 8th day
3	III	„	„	„	do. do.
4	I	„	Type I antitoxin		General tetanus 10th day
5	II	„	„	„	Animal remained well
6	III	„	„	„	General tetanus 10th day
7	I	„	Type II antitoxin		General tetanus 10th day
8	II	„	„	„	Animal remained well
9	III	„	„	„	General tetanus 9th day

This experiment is really valueless, for the time which elapsed between making the inoculation and the onset of the disease in the control animals Nos. 1, 2, and 3, was so long, that the passive immunity in the experimental animals must have almost disappeared.

I would call attention to the fact, that both animals 5 and 8 remained well. This suggested that Experiment VIII should be accepted with a certain reserve, as it appeared possible, that the infectivity of the Type II spores used might be somewhat less than that of the Type I and Type III spores. In view of the result of Experiment IX, I do not think that this was the



case in Experiment VIII, but the possibility of such an interpretation of the results at any stage of the investigation, must be borne in mind.

The experiment was then repeated, and on this occasion sufficient *Welchii* toxin was administered to cause swelling of the whole of the inoculated limb. The result was that none of the animals survived, and the protected animals took the disease almost as soon as the control animals. These tests on guinea-pigs and three other tests which were carried out on mice, convinced me, that one could not hope to obtain consistent results if *Welchii* toxin were employed as the tissue debilitating factor in the mixtures, owing to the difficulty of regulating the dose.

*Note.* Experiments VIII, IX, and X, and their repetitions were carried out with sample bleedings—two different samples were used—from Rabbit I', while the animal was still undergoing immunisation. Before the next series of tests was made the animal had been bled out.

It was at this stage of the investigation that I decided to change my technique, and in guinea-pigs at least, to employ chemical reagents as the tissue debilitating factor of the infecting mixtures.

Several chemical agents were tried, but saponin gave the most constant results, and was fairly easily handled, so that this substance was chosen for future investigations.

#### B. EXPERIMENTS IN WHICH SAPONIN WAS USED AS THE TISSUE DEBILITANT AND THE ANTI-INFECTIVE QUALITY OF ANTITOXIN WAS UNDER INVESTIGATION.

##### *Experiment XI.*

In this experiment the animals were passively immunised with 25 units of antitoxin, and two days later, were inoculated by the intramuscular route with a mixture of saponin and spores.

- (a) Animals = guinea-pigs.
- (b) Type I antitoxin = B.W. Horse 13.
- (c) „ II „ = Rabbit I'.
- (d) Antitoxin dose = 25 units.
- (e) „ administered 48 hours before inoculations were made.
- (f) Spores = 200 million.
- (g) Saponin = 0.2 c.c. of 1/200 dilution.
- (h) Volume of infecting *inoculum* = 0.6 c.c.

Animal No.	Spores Type	Saponin	Antitoxin units 25	Result
1	I	0.2 c.c. 1/200	Nil—saline only	Generalised tetanus 2nd day
2	II	„	„ „	„ „ „
3	III	„	„ „	„ „ „
4	I	„	Type I	Generalised tetanus 11th day
5	II	„	„	Generalised tetanus 3rd day
6	III	„	„	„ „ „
7	I	„	Type II	„ „ „
8	II	„	„	„ „ „
9	III	„	„	„ „ „

The experiment was then performed by the method of making simultaneous inoculation of serum, debilitant and spores. The details are as follows.

*Experiment XII.*

- (a) Animals = guinea-pigs.
- (b) Spores = 200 million.
- (c) Saponin = 0.1 c.c. of 1/100.
- (d) Antitoxin = 5 units.
- (e) Total volume of *inoculum* = 0.6 c.c.
- (f) Inoculation of antitoxin and infecting mixture made simultaneously.
- (g) Antitoxic sera employed were the same as in Experiment XI.

Animal No.	Spores Type	Saponin	Antitoxin 5 units	Result
1	I	0.1 c.c. of 1/100	Nil—saline only	Generalised tetanus 2nd day
2	II	„	„ „	„ „ „
3	III	„	„ „	„ „ „
4	I	„	Type I	Animal developed local tetanus 14th, generalised 15th day
5	II	„	„	Generalised tetanus 3rd day
6	III	„	„	„ „ „
7	I	„	Type II	Generalised tetanus 2nd day
8	II	„	„	„ „ 4th „
9	III	„	„	„ „ 2nd „

That portion of the experiment dealing with Type II antitoxin was repeated twice as the above result did not corroborate Experiments VIII and IX in which *Welchii* toxin had been used as the tissue debilitant. Both experiments failed to show definite protection against infection; either *general*, in relation to all the Types, or specific, in respect of the *homologous* Type.

Examination of this serum showed that it was contaminated with a diplococcus. The serum was filtered, and the experiments were discontinued until such time as complete crossed experiments could be performed, using both *anti-bacterial* and *antitoxic* sera.

In these experiments the following points call for comment:

1. I failed to corroborate the findings of Experiment VIII in respect of Type II serum, both when the serum was administered before inoculation of the infecting mixture and when it was administered simultaneously therewith.

2. It is significant, on the other hand, that Type I antitoxin did protect for a much longer period against infection with Type I spores, than did Type II antitoxin.

3. It is remarkable that a dose of 5 units of antitoxin, when administered simultaneously with the infecting mixture, appears to give almost as much protection against infection, as does a dose of 25 units of the same antitoxin administered two days prior to the inoculation of the infecting mixture.

Before leaving this subject I decided to repeat Experiment VIII, using Type I antitoxin, and saponin as the irritant. My reason for so doing was, that in neither Experiment XI nor XII did I succeed in obtaining an unequivocal result. In performing this further experiment, the test animals

were passively immunised with 50 units of antitoxin and were inoculated with the saponin-spore mixture two days later.

### Experiment XIII.

- (a) Animals = guinea-pigs.
- (b) Spores = 200 million.
- (c) Saponin = 0.1 c.c. of 1/100 dilution.
- (d) Volume of *inoculum* = 0.6 c.c.
- (e) Antitoxin used = B.W. Horse 13.
- (f) Dose of antitoxin = 50 units.
- (g) Antitoxin administered two days before infecting inoculations were made.

Animal No.	Spores Type	Saponin	Antitoxin 50 units	Result
1	I	0.1 c.c. of 1/100	Nil—saline only	General tetanus 2nd day
2	II	„	„ „	„ „ „
3	III	„	„ „	„ „ „
4	I	„	Type 1 B.W. 13	Remained well till 12th day, died 13th, streptococci recovered from local lesions and internal organs
5	II	„	„	Local tetanus 4th day, generalised 8th day
6	III	„	„	Local tetanus 4th day, generalised 5th day

The following deductions seem permissible from experiments VIII–XIII, pp. 155–160:

1. That *antitoxic* serum may under certain conditions exhibit *anti-infective* properties; and

2. While the experiments afford no *proof* that a monotypical antitoxic serum protects more adequately against *infection* due to serologically *homologous* strains of the bacillus than against that due to *heterologous* strains, the results obtained nevertheless *suggest* that protection against the *homologue* is more adequate.

These deductions are made in a spirit of modesty, for one is well aware that, in infection experiments, and especially with the anaërobes, even the known factors upon which the process depends are very complex. Short of obtaining a 100 per cent. result in a considerable series of experiments, it is rash to conclude that proof of any thesis has been established.

*Does serum possessing anti-bacterial properties exhibit more adequate prophylactic qualities than serum which possesses only antitoxic (anti-spasmin) properties?*

*Note.* While the term “unit” as previously employed refers to the U.S.A. Unit, I wish especially to call attention to the fact that, in the experiments of the *following Sub-section*, the term “unit” is defined as “that quantity of antitoxin which will protect a mouse against 1000 M.L.D. of tetanus toxin.”



The investigation of this problem involved an inquiry into the following points:

(i) Does serum possessing demonstrable *anti-bacterial* qualities (e.g. agglutinin and phagocyte stimulating properties) but of no, or only negligible antitoxic value, exhibit any capacity for preventing *infection*?

(ii) We know that *antitoxin* has a certain *anti-infective* power. Can this be enhanced by adding serum containing *anti-bacterial* immune bodies? If antitoxic (anti-spasmin) content be made the basis of assessing the probable prophylactic value of sera, does a serum of  $x$  "anti-spasmin" units which has no *anti-bacterial* qualities, protect against infection as effectively as a serum of  $x$  "anti-spasmin" units, which does possess *anti-bacterial* properties?

(iii) Can specific protection be demonstrated *in vivo* with such *anti-bacterial* sera?

The consideration of these points is of extreme import; for, should any of these questions be answered in the affirmative, the method of standardising prophylactic sera will demand review. It is only rational that, if a satisfactory method could be devised for examining sera designed for prophylactic use, these sera should be standardised on the basis of prevention of *infection*, rather than of *intoxication*.

The first experiments made were designed to corroborate the findings already obtained, and to determine a method of procedure which could be satisfactorily used for the purpose in view.

#### Experiment XIV.

This experiment was carried out as a preliminary to more careful and exact tests, in which guinea-pigs were to be used. In this instance mice were the experimental animals employed. The tissue irritant chosen was therefore *Welchii* toxin and a complete series of crossed tests was made, both with monotypical *antitoxic* and monotypical *anti-bacterial* sera corresponding to three serological Types of *B. tetani*.

The details of the experiments are as follows:

- (a) Animals = mice.
- (b) Tissue irritant = *Welchii* toxin  $< 1/2$  but  $> 1/4$  M.L.D.
- (c) Spores = 100 million.
- (d) Inoculation = *Sub cutem*.
- (e) *Antitoxic* sera dose = 1 "unit."
- (f) *Anti-bacterial* sera dose = antitoxic content of  $< 1$  "unit" and  $> 1/2$  "unit."
- (g) Sera administered one day prior to inoculation of the infecting mixtures.
- (h) Volume of infecting *inoculum* = 0.2 c.c.

#### Type I Spores.

1.	<i>Welchii</i> toxin nil	Serum nil	Died 5th day, no evidence of tetanus
2.	<i>Welchii</i> toxin	+ serum nil	Remained well
3.	" "	+ Type I <i>antitoxin</i>	" "
4.	" "	+ " II "	Died 2nd day—? tetanus
5.	" "	+ " III "	" " "
6.	" "	+ " I <i>anti-bacterial</i>	Remained well
7.	" "	+ " II "	" "
8.	" "	+ " III "	Died 2nd day—? tetanus

## Type II Spores.

1. <i>Welchii</i> toxin nil	Serum nil	Remained well
2. <i>Welchii</i> toxin	"	Definite tetanus 3rd day, died
3. " "	+ Type I <i>antitoxic</i> serum	Definite tetanus 4th day
4. " "	+ " II " "	Remained well
5. " "	+ " III " "	" "
6. " "	+ " I <i>anti-bacterial</i> serum	" "
7. " "	+ " II " "	" "
8. " "	+ " III " "	Definite tetanus 3rd day

## Type III Spores.

1. Nil	Nil	Died 4th day, no evidence of tetanus
2. <i>Welchii</i> toxin	"	Definite tetanus 3rd day
3. " "	+ Type I <i>antitoxic</i> serum	" "
4. " "	+ " II " "	" "
5. " "	+ " III " "	Remained well
6. " "	+ " I <i>anti-bacterial</i> serum	Died 3rd day, ? evidence of tetanus
7. " "	+ " II " "	Remained well
8. " "	+ " III " "	" "

The results of Experiment XIV may be summarised thus:

(i) Of those animals which received spores *homologous* to antitoxic sera employed, all three remained well.

(ii) Of six which received spores *heterologous* to the antitoxin three developed definite tetanus and died; two others died but evidence of death from tetanus was lacking, and one remained well.

(iii) Of nine animals which received *anti-bacterial* sera, one showed definite tetanus; two died but evidence of tetanus was lacking, and six remained well.

Considering that the unitage of the *anti-bacterial* sera in the above experiment was certainly less than that of the *antitoxic* sera, it is notable that so many survivals occurred among the animals protected with the former.

The section of the experiment which deals with Type I spores is, however, really valueless, in that infection did not "take" in the control.

If then we consider only those sections of this experiment which deal with Type II and Type III spores, it is seen that

(a) Of six mice protected with *antitoxic* sera, three developed tetanus and three remained well.

Two of the surviving mice were protected by sera *homologous* to the infecting organisms; in one the infection was *heterologous*.

(b) Of six animals protected by *anti-bacterial* sera, only one developed tetanus and four remained well. One mouse died on the third day after inoculation but evidence of death from tetanus was lacking. Both of those in which the sera and the infecting organisms were *homologous* were included among the four mice which remained well.

Here again is a suggestion, but no proof, that *monotypical* antitoxic sera may, under certain circumstances, exhibit a specific influence on infection from *serologically homologous* bacilli.



(c) There is also an indication that *anti-bacterial* sera exhibit more adequate *anti-infective* properties than do *antitoxic* sera, provided that the "anti-spasmin" value of the sera be made the basis of comparison.

*Note.* There is some ground for believing that mice are not very susceptible to tetanus *infection*. When these animals are employed, the assessment of the results obtained is rendered especially difficult.

Far-reaching conclusions cannot be drawn from the results obtained.

The above experiment, which lays no claim to strict accuracy, was valuable however, in that it served to indicate how to proceed further and showed that the thesis advanced was worthy of extended investigation.

Henceforth guinea-pigs only were employed as the experimental animals, and the irritant used was saponin. More reliable results could be expected with this technique, than when mice and *Welchii* toxin were used.

### Experiment XV.

This experiment was carried out to determine whether *anti-bacterial* sera, possessing little or no antitoxic properties, would protect against *infection*. Sample bleedings were made from Rabbits III, III' and III'', "anti-whole culture" serum to Types I, II and III respectively. These sera, retained from a previous investigation, showed some phagocytic properties when diluted 1/200, and their antitoxic value was equal to about 2 "units" per c.c. With these serum samples the following experiment was carried out.

- (a) Animals = guinea-pigs.
- (b) Spores = 200 million.
- (c) Tissue irritant = 0.1 c.c. of 1/100 saponin.
- (d) Inoculation = intramuscular.
- (e) *Anti-bacterial* sera Type I, Type II and Type III (equivalent of 1 "anti-spasmin" "unit" used).
- (f) *Antitoxic* serum = Type I (equivalent of 6 "anti-spasmin" "units" used).
- (g) Sera administered one day prior to inoculation of infecting mixture.
- (h) Volume of infecting *inoculum* = 0.6 c.c.

Animal No.	Spores	Serum	Result
1	I	Nil	General tetanus 4th day
2	II	"	" " 3rd "
3	III	"	Animal remained well
4	I	6 "units" Type I <i>antitoxic</i>	General tetanus 7th day
5	II	" " "	" " 3rd "
6	III	" " "	" " 7th "
7	I	1 "unit" Type I <i>anti-bacterial</i>	" " 3rd "
8	II	" " "	" " 3rd "
9	III	" " "	" " 3rd "
10	I	1 "unit" Type II <i>anti-bacterial</i>	" " 4th "
11	II	" " "	" " 2nd "
12	III	" " "	" " 3rd "
13	I	1 "unit" Type III <i>anti-bacterial</i>	" " 3rd "
14	II	" " "	" " 2nd "
15	III	" " "	" " 3rd "



Experiment XV shows that sera which possess *anti-bacterial* properties will not serve to prevent a fatal issue in an experimental infection set up by the method described, if the sera do not also possess sufficient *antitoxic* qualities.

This fact was confirmed by investigations made with agglutinating sera which possessed no, or negligible *antitoxic* qualities. These also failed to prevent infection under the conditions of the experiment.

I wish to call attention to the survival of the control animal which received Type III spores. In view of such survivals, extreme care must be exercised in drawing conclusions from the results of these tests.

Experiment XV was repeated with a modified technique; serum and infective material being injected simultaneously, and only *anti-bacterial* sera used. The quantity of each serum used contained 1 "unit" of antitoxin.

The result of this experiment showed, that even under these conditions, the *anti-bacterial* sera did not exert demonstrable *anti-infective* qualities in the experimental infection set up by the method described.

This result was somewhat unexpected; but, when it is borne in mind that saponin has an extremely deleterious influence on all the tissues, and is very markedly haemotoxic, this can be readily understood. For, during the first day or two after inoculation, *B. tetani* develops virtually outside the body, and may elaborate enough toxin to kill the animal before sufficient local reaction is set up in the tissues to deal with the process of infection. If it were possible to combat this initial intoxication, and at the same time favourably influence the tissues to deal with the local infective process, better results might be obtained.

With this object in view the following experiment was undertaken.

Experiment XVI.

Four guinea-pigs, A, B, C and D, each received 20 "units" of Type III antitoxin (*i.e.* antitoxin prepared by inoculation of the toxin of Type III bacilli).

The following day

A was inoculated with a mixture of saponin and Type I spores.					
B	"	"	"	"	I "
C	"	"	"	"	III "
D	"	"	"	"	III "

Two days were allowed to elapse and then,

A was given a dose of 2 "units" of Type III <i>anti-bacterial</i> serum.					
B	"	"	2	"	<i>antitoxic</i> "
C	"	"	2	"	<i>anti-bacterial</i> "
D	"	"	2	"	<i>antitoxic</i> "

Animals A, C and D remained well, while B developed generalised tetanus

six days after the inoculation of the infective mixture. In this experiment then,

- (a) The animal which received *homologous* antitoxin survived.
- (b) The animal which received only *heterologous* antitoxin died from tetanus.
- (c) Both animals, which on the second day after inoculation of infective material received a dose of *anti-bacterial* serum, recovered.

In this experiment too large an initial dose of antitoxin was used to permit of its being satisfactorily demonstrated that *anti-bacterial* sera possess more adequate *anti-infective* properties than do *antitoxic* sera.

In Experiment XVII an effort was made to overcome this difficulty by using a smaller dose of serum.

#### *Experiment XVII.*

Four guinea-pigs A, B, C and D were passively immunised as follows:

A received 12 "units" of Type III *antitoxic* serum

B    "     6       "       "       "       + 4 "units" of Type III *anti-bacterial* serum

C    "     12       "       "       *antitoxic* serum

D    "     6       "       "       "       + 4 "units" of *anti-bacterial* serum

The following day A and B were each inoculated intramuscularly with a mixture of saponin 0.1 c.c. of 1/100 and Type I spores 200 million, total volume 0.6 c.c. C and D each received a similar inoculation, but with Type III instead of Type I spores.

Animal A developed local tetanus on the third day after inoculation, the disease being generalised on the fifth day.

„ B developed local tetanus on the fifth day which generalised on the sixth day.

„ C developed local tetanus on the third day, general tetanus supervening on the following day.

„ D remained well.

This experiment definitely showed that *anti-bacterial* sera were worthy of extended investigation.

I would call attention to the fact, that Experiments XVI and XVII illustrate a further difficulty, which has so far not been overcome, viz. that the bacilli, from a given number of spores, are not always equally toxogenic. In Experiment XVI, Animal B (inoculated with Type I spores), although it received in all 22 "units" of antitoxin, developed generalised tetanus six days after inoculation. Whereas in Experiment XVII when the same spores, but only half the quantity of the same antitoxin, were used, generalised tetanus developed only one day earlier—the fifth day—preceded by local tetanus for a period of two days. In Experiment XVII then, the Type I spores did not appear to be so toxogenic as in Experiment XVI.

In order, finally, to examine this subject, a complete series of experiments was carried out. Great care was taken to standardise the reagents as accurately as the equipment of my laboratory would permit, and to carry out the experiments under standard conditions as far as possible.

The following are the protocols of experiments carried out with the specimen



bleedings of *antitoxic* and *anti-bacterial* sera. The sera were standardised with fair accuracy before the tests were performed.

As it was not possible to carry out the entire experiment with all six sera—three *antitoxic* and three *anti-bacterial*—on the same day, the experiment had to be divided into three sections. Each section deals with the investigation of the *anti-infective* value of all the sera in respect of only one Type of spores.

#### METHODS USED.

(1) The basis of comparison for the sera was their *antitoxic* value. This was standardised as accurately as possible; but no doubt slight differences existed between the real and the estimated unitage of each. I was unable to obtain any standard U.S.A. toxin with which to perform the preliminary tests and therefore had to adhere to the "mouse M.L.D." method of standardisation. Such differences, however, do not really affect the results, as the same ratio obtains in each experiment of the series.

(2) The quantity of antitoxin provisionally decided upon was 10 units.

(3) The sera were injected *sub-cutem* into the right hind limb.

(4) One hour after the administration of the sera, a mixture consisting of 0.1 c.c. 1/100 dilution of saponin + 200 million spores suspended in 0.6 c.c. of saline, was injected intramuscularly into the left hind limb.

(5) The animals employed were guinea-pigs.

(6) The volume of serum administered in each instance was made up to 2 c.c. by the addition of normal rabbit serum. (The *antitoxic* and *anti-bacterial* sera used in these experiments were obtained from later bleedings from the same rabbits as were used in the experiments described in Section IV.)

(7) The site of the inoculation where the saponin spore mixture was injected was dried with alcohol and sealed with collodion. This precaution is essential, because of the danger of secondary infection from the animal cage, bedding, etc. This danger is enhanced by the tissue devitalisation produced by the saponin.

#### Experiment XVIII.

(a) Type I spores used.

(b) Antitoxic titre of each serum tested—10 "units."

Number	Serum	Result
1	Normal rabbit serum 2 c.c.	Died from tetanus 3 days after inoculation
2	Type I <i>antitoxic</i> serum 10 "units"	Local tetanus developed 3rd day; persisted for 8 days. Thereafter the animal slowly recovered
3	Type II <i>antitoxic</i> serum 10 "units"	Local tetanus developed 3rd day; persisted for over three weeks
4	Type III <i>antitoxic</i> serum 10 "units"	Local tetanus 3rd day; generalised on 4th; animal killed
5	Type I <i>anti-bacterial</i> serum 10 "units"	Animal remained well
6	Type II <i>anti-bacterial</i> serum 10 "units"	Local tetanus 3rd day; generalised 4th; animal killed
7	Type III <i>anti-bacterial</i> serum 10 "units"	Local tetanus 4th day; generalised 5th; animal killed



*Experiment XIX.*

(a) Type II spores used.

(b) Antitoxic content of each serum—7.5 “units.” (In view of the survival of animals 2 and 3 in Experiment XVIII the smaller dose—7.5 “units of” antitoxin—was decided upon.)

Number	Serum	Result
1	Normal rabbit serum 2 c.c.	Died from tetanus 3 days after inoculation
2	Type I <i>antitoxin</i> 7.5 “units”	Local tetanus 3rd day; generalised 4th; animal killed
3	Type II <i>antitoxin</i> 7.5 “units”	Local tetanus 3rd day; generalised 4th; animal killed
4	Type III <i>antitoxin</i> 7.5 “units”	Local tetanus 3rd day; both hind legs tetanised from 5th–12th day; animal commenced to use both hind limbs 17th day; recovered
5	Type I <i>anti-bacterial</i> serum 7.5 “units”	Local tetanus 3rd day; generalised 4th; animal killed
6	Type II <i>anti-bacterial</i> serum 7.5 “units”	Remained well
7	Type III <i>anti-bacterial</i> serum 7.5 “units”	Local tetanus 3rd day; generalised 4th; animal killed

*Experiment XX.*

(a) Type III spores used.

(b) Antitoxic content of each serum—10 “units.”

[The higher dose of antitoxin was reverted to in this instance, as previous experiments with Type III spores had indicated that these were probably markedly toxogenic.]

Number	Serum	Result
1	Normal rabbit serum 2 c.c.	Local tetanus 2nd day; generalised tetanus 3rd day; killed
2	Type I <i>antitoxin</i> 10 “units”	Local tetanus 2nd day; generalised tetanus 3rd day; killed
3	Type II <i>antitoxin</i> 10 “units”	Local tetanus 3rd day; did not generalise; remained for 10 days; thereafter animal slowly recovered
4	Type III <i>antitoxin</i> 10 “units”	Local tetanus 3rd day; did not generalise; remained for 10 days; thereafter animal slowly recovered
5	Type I <i>anti-bacterial</i> serum 10 “units”	Local tetanus 3rd day; did not generalise; remained for 8 days; thereafter slowly recovered
6	Type II <i>anti-bacterial</i> serum 10 “units”	Local tetanus 3rd day; generalised 4th day; animal killed
7	Type III <i>anti-bacterial</i> serum 10 “units”	Local tetanus 3rd day; generalised 4th day; animal killed

This result fails to corroborate Experiments XVIII and XIX. Animals 3 and 5, although only immunised with *heterologous* sera, survived for a longer period than the animal which received *homologous anti-bacterial* serum.

It is to be noted that animals 3, 4 and 5 were all very small guinea-pigs; they exhibited the same markings, and appear to have been, though the evidence is not definite, from the same litter. It has been noted throughout the whole series of infection experiments that small guinea-pigs are

less susceptible and give less constant results than larger animals. This probably because it is more difficult to ensure an intramuscular injection being made when the animals are small. Attention is therefore especially called to the fact that these animals which survived in Experiments XVIII and XIX were comparable to those which died in Experiment XX. Owing to lack of animals, I was unable to obtain nine which were really comparable to one another.

A further series of experiments was therefore carried out using larger animals. The animals in each test were of approximately equal weight.

Owing to a shortage of guinea-pigs these tests were confined to an examination of three sera only, each serum being examined in triplicate, so that a range of unitage could be employed in the investigation of each.

The sera examined were:

- (a) *Antitoxic* serum = Type III.
- (b) *Anti-bacterial* serum = Type II.
- (c) *Anti-bacterial* serum = Type III.

### Experiment XXI.

The details of this experiment are as follows:

- (a) Animals = guinea-pigs of approximately 350 grams.
- (b) Spores = Type III—200 million.
- (c) *Anti-spasmin* "unitage" in each case, 9, 12 and 15.
- (d) Irritant = Saponin, 1/100, 0.1 c.c.
- (e) Volume of *inoculum* = 0.6 c.c.
- (f) Serum administered one hour before injection of spore-saponin mixture.

In this experiment, the *anti-bacterial* value of the sera employed remained constant in each test, the equivalent of 6 "units." The balance of *antitoxin* was made up of 3, 6 and 9 U.S.A. Units of a polyvalent antitoxic horse serum marked L.I.P.M. 136 A.

The following were the results obtained

Animal No.	Serum administered	Spores	Result		
		Type III	General tetanus 2nd day		
1	6 "units" Type III <i>antitoxin</i> (rabbit), 3 units L.I.P.M. 136 A				
2	6 "units" Type III <i>antitoxin</i> (rabbit), 6 units L.I.P.M. 136 A	"	"	"	"
3	6 "units" Type III <i>antitoxin</i> (rabbit), 9 units L.I.P.M. 136 A	"	"	"	"
4	6 "units" Type II (rabbit) <i>anti-bacterial</i> , 3 units L.I.P.M. 136 A	"	"	"	"
5	6 "units" Type II (rabbit) <i>anti-bacterial</i> , 6 units L.I.P.M. 136 A	"	"	"	"
6	6 "units" Type II (rabbit) <i>anti-bacterial</i> , 9 units L.I.P.M. 136 A	"	"	"	"
7	6 "units" Type III (rabbit) <i>anti-bacterial</i> , 3 units L.I.P.M. 136 A	"	"	"	"
8	6 "units" Type III (rabbit) <i>anti-bacterial</i> , 6 units L.I.P.M. 136 A	"	"	"	"
9	6 "units" Type III (rabbit) <i>anti-bacterial</i> , 9 units L.I.P.M. 136 A	"	Local tetanus 2nd day General tetanus 4th day		

This experiment will be commented on later; but I wish to call attention to the somewhat longer survival of animal 9 as compared with animals 3 and 6.

### *Experiment XXII.*

In this experiment though similar to the previous one horse serum was not added to bring up the desired *anti-spasmin* content of the *anti-bacterial* sera. Both *anti-spasmin* and *anti-bacterial* content therefore varied *pari passu* one with the other.

The details of the test are substantially the same as those of Experiment XXI but 10, 20 and 30 "units" were employed instead of 9, 12 and 15.

Animal No.	Serum administered	Spores	Result
1	Type III rabbit <i>antitoxin</i> 10 "units"	Type III	General tetanus 2nd day
2	Type III rabbit <i>antitoxin</i> 20 "units"	"	" " "
3	Type III rabbit <i>antitoxin</i> 30 "units"	"	" " 3rd day
4	Type II rabbit <i>anti-bacterial</i> 10 "units"	"	" " 2nd "
5	Type II rabbit <i>anti-bacterial</i> 20 "units"	"	" " "
6	Type II rabbit <i>anti-bacterial</i> 30 "units"	"	Local tetanus 3rd day General tetanus 4th day
7	Type III rabbit <i>anti-bacterial</i> 10 "units"	"	Local tetanus 2nd day General tetanus 3rd day
8	Type III rabbit <i>anti-bacterial</i> 20 "units"	"	Local tetanus 2nd day General tetanus 3rd day
9	Type III rabbit <i>anti-bacterial</i> 30 "units"	"	Local tetanus 3rd day General tetanus 4th day

I shall not comment on these results beyond calling attention to the fact, that it is obvious, that if specificity of reaction between serum and organism exists, it is obviously *quantitative*. Experiments XVIII, XIX, XX, XXI and XXII all show the difficulty of realising the conditions necessary for the demonstration of specificity.

### SYNOPSIS OF RESULTS OBTAINED IN SECTION V (pp. 135-169).

The results obtained in the Section of the Report at present under consideration may be summarised thus:

1. The genesis of tetanus *infection* induced by experimental methods depends very largely upon the degree and extent of the tissue debility produced by the *inoculum*.

2. The nature of the tissue debilitant employed for setting up infection is an important factor in this connection; for a debilitant which lights up infection in one animal species may fail to do so in another. Thus, when mice are inoculated with mixtures of saponin and tetanus spores, these animals only occasionally develop the disease. On the contrary, when guinea-pigs are inoculated with such mixtures they seldom survive, the onset of the disease being usually early and its character fulminant.

3. The quality of the tissue debility induced is also of paramount impor-



tance in the genesis of infection. Thus trimethylamine, even though it be used in sufficient concentration to cause the development of large eschars at the site of inoculation, when injected together with tetanus spores into mice only infrequently lights up the disease; whereas the toxin of *B. Welchii*, injected in sub-lethal dose together with the same spores, almost invariably sets up tetanus in these animals, notwithstanding the fact that no obvious lesion could be demonstrated *ante-mortem*.

4. In animals protected by *antitoxin*, a rapidly fatal tetanus infection can be induced, provided that the spore-containing *inoculum* produce a sufficient degree of tissue debility of the requisite character.

5. In the case of infections arising from Type I and Type II spores (Type III spores were not included in the test), it was shown that in guinea-pigs, by increasing the prophylactic dose of *antitoxic* serum, the onset of the disease can be delayed and its course can be rendered less acute. If the increase of prophylactic dose be sufficient, *antitoxic* serum *may* completely protect these animals against an *infection* induced by the inoculation of "saponin-spore" mixture.

6. There is some evidence that *monotypical antitoxic* sera protect more adequately against infection with bacilli which are serologically *homologous*, than against infection due to bacilli which are serologically *heterologous*.

Admittedly, the experimental evidence of this is only suggestive, and this deduction is made with great reserve.

7. The experiments on the relative prophylactic value of *antitoxic* and *anti-bacterial* sera prove that this subject is worthy of careful investigation.

8. Experiments XVIII and XIX (pp. 166, 167) in which Type I and Type II spores were used, strongly suggest that sera possessing *anti-bacterial* as well as *antitoxic* properties protect more adequately than do sera exhibiting only *antitoxic* properties. The evidence of specific protection in relation to the serological Type of the infecting organism appears also to be definite in these two experiments.

9. Experiment XX (p. 167), in which Type III spores were used, failed to corroborate this finding. The experiment is however interesting, in that it indicates that there is a considerable variation of susceptibility to tetanus infection among animals of one species.

10. Experiments XXI and XXII (pp. 168, 169) give a very slight indication that in the case of Type III also, *anti-bacterial* serum affords more adequate protection than does *antitoxic* serum. There is also a suggestion in the results obtained that the protection is specific.

#### DISCUSSION OF THE RESULTS OF EXPERIMENTS OF SECTION V.

It is difficult to comment upon the results obtained in Section V, for, although suggestive, they fall very far short of establishing the validity of the thesis advanced. I wish then formally to call attention to the fact that

any deductions which have been drawn from the results so far obtained, are to be regarded as only provisional in character.

The following criticisms are applicable to the experiments described.

(a) The number of experiments performed is too small to permit of definite information being obtained on the points at issue.

(b) The method employed for setting up infection is unsatisfactory for the following reasons.

(i) To set up tissue destruction or debility by means of saponin is a highly artificial procedure.

(ii) The disturbance, which must be produced when this reagent is the debilitant employed, is so great (if infection in the control animals is to be assured), that it is extremely difficult to protect against infection, even when relatively enormous doses of serum are used prophylactically.

This large amount of tissue destruction really means that the inoculated spores develop in a situation relatively remote from the body-fluids, and in such circumstances may be protected from immune bodies circulating in the blood or lymph. As the germinated bacilli continue to develop toxin, any antitoxin that is administered may ultimately be exhausted, and tetanus supervenes.

The method which one is forced to employ has therefore grave disadvantages, and leads, probably, to too severe a test being applied to the sera examined.

(iii) It is obvious that a standard method of infection is an unattainable ideal, for spores certainly vary in respect both of their *toxogenic* capacity and of their *infective* capacity.

It is to be noted that the two factors of *toxogenicity* and *infectivity* are not identical, for the spores from some cultures which have a relatively low toxin content may, under certain circumstances, be more highly *infective* than are spores from a culture of greater toxicity.

(iv) When all other sources of error which render comparative experiments of the kind under consideration difficult to carry out are eliminated, there remain two factors which never can be standardised.

(a) The test animals will always exhibit idiosyncrasy.

(b) The *toxogenic* capacity, and also probably the *infective* capacity, of a single strain of *B. tetani* varies from culture to culture.

A very important question arises in connection with this section of the work, viz. "Can we hope for any improvement in the serotherapeutics of declared tetanus by the use of *anti-bacterial* sera?"

While it is unfortunately probable that this question will have to be answered in the negative, experiments dealing with the problem will have to be undertaken.

These experiments cannot be carried out until larger quantities of *anti-bacterial* sera are available; but arrangements are now being made for the immunisation of horses against *whole culture*. The problem will



therefore be dealt with when sufficient serum from these animals is obtained.

Since completing the experiments described in Section V, I have received information, communicated personally, from Capt. Bullock, R.A.M.C., Imperial Cancer Research Laboratories, concerning the influence of the calcium ion in initiating anaërobic infections.

I here wish to record my very deep debt to Capt. Bullock, R.A.M.C., for the information which he has placed unreservedly at my disposal before the publication of his work.

Preliminary experiments with soluble calcium salts as the "Infection initiator" both in mice and in guinea-pigs have given encouraging results; and, in view of the slight tissue destruction which occurs when calcium salts are used for this purpose, I propose to repeat Experiments XVIII, XIX and XX using Bullock's technique.

## SECTION VI.

### INVESTIGATION OF DRESSINGS IN RELATION TO ANAËROBIC INFECTIONS OF WOUNDS.

In this section of the Report are considered the results obtained in the examination of 100 wounds during the process of healing.

The object of the inquiry was to determine, if possible, the procedure or procedures, which might be recommended as useful for preventing or diminishing infection of wounds due to anaërobic bacteria.

In view of the influence which anaërobes have one upon another, the general question of all anaërobic infection, rather than the particular question of any one such infection, was investigated. The presence of *B. tetani* was, however, made the subject of detailed inquiry, as the prevalence of the various serological Types of that organism, and its incidence in wounds, constitute a most important aspect of the bacteriology of tetanus.

Owing to its being relatively easy to differentiate morphologically, and because also of its important relationship to the etiology of tetanus, the incidence of *B. Welchii* was also noted. No effort could be made, however, to determine the incidence of other anaërobes in the material examined.

#### (a) *Methods used.*

It was felt, that in making this investigation, it would be better to examine thoroughly a relatively small number of wounds, and to examine them repeatedly, than to make a single examination of a much larger number. The progress made under the various treatments could be more definitely assessed by choosing a small number of cases, that were more or less comparable to one another; that is, in so far as comparison of wounds is at all possible. Five examinations of each wound were made at weekly intervals,



unless the patient became convalescent during the period. The examinations were conducted as follows:

(i) Swabs were taken as soon after the arrival of a convoy as possible.

(ii) Thereafter the same wounds were examined at intervals of approximately seven days, for a period of five weeks.

The material of each swab was inoculated into meat water tubes—"a" and "b." The tubes were boiled prior to inoculation, in order to ensure anaërobiosis.

Tube "a" was incubated anaërobically to obtain a growth of non-sporulating organisms.

Tube "b" was heated to 65° C. for 30 minutes before incubation, in order to obtain growths of sporing anaërobes only.

Each culture was examined ten times at intervals of three or four days.

When the anaërobes developed rapidly, and in some variety, the swab was marked in the records as "heavily infected"; and when the cultures showed only a slight or medium growth, the result was noted as "light anaërobe infection."

It might be argued that a notation of this kind is arbitrary, but only by taking a broad view, could one preserve a correct perspective of the results obtained. In Diagrams XIII, XIV, XV, XVI and XVII, pp. 191-195, are given the details of the examination of each swab.

(iii) When organisms, having the morphological characters of *B. tetani*, appeared in the meat cultures, these were sub-cultured into the "exhausted" medium described in previous communications, and the growth therefrom tested by the agglutination methods. When a culture was obtained that agglutinated in the presence of any of the type sera, its toxogenicity was then tested by sub-culture from the original meat tube, and, in certain instances, its infective quality was also examined.

(iv) The tubes "a," incubated without previous heating, were examined with a view to demonstrating the presence of *B. Welchii*.

*Note.* This procedure was only introduced after swabs from 36 cases had already been examined. The figures relating to *B. Welchii* deal, therefore, with only 64 cases.

It is specially to be noted, that these swabs were always taken by one observer and we wish to record our debt of gratitude to Miss Smithwhite who was in charge of this section of the work. Her enthusiasm and care in collecting material, the attention devoted to keeping detailed and accurate records of each case, and the extracting of the necessary information from the field cards of the cases investigated—duty demanding much expenditure of time and labour—have greatly assisted in the prosecution of the work.

(b) *Inherent fallacies incidental to all bacteriological inquiries dealing with the treatment of wounds.*

In addition to the difficulties that are encountered in making an inquiry into the bacterial flora of wounds, the problem under consideration presented its own peculiar difficulties; of these the following may be cited.

(i) The diversity of methods of treatment in use both in France and in England is such that, to obtain a sufficiency of observations on which to base definite conclusions concerning any one method, would involve not months, but years of study.

(ii) It is well nigh impossible to get continuity of method in France and in England.

(iii) While a certain number of cases are evacuated to England, and find their way to hospitals in a single district in this country within a week or two after the soldiers have been wounded, the majority of wounds are not seen on this side until considerably later than this. Herein arises an almost insuperable difficulty, viz. that men whose wounds are slow to heal are sent to England, with the result that a special type of wound, showing chronic infection with a variety of organisms, both anaërobic and aërobic, is liable to predominate in a series of wounds examined in home hospitals.

(iv) Apart from these influences, there is the fact, that owing to idiosyncrasy of certain of the patients, some wounds will heal, not because of, but one might almost say in spite of, treatment; whereas others, apparently comparable to them, cannot be induced to heal under any treatment. The two cardinal points, which must not be lost sight of, are:

(a) That surgery is more an art than a science, therefore more depends upon the surgeon in charge of the case than on the dressing which he employs.

(b) That the best protection which the tissues can have against bacterial invasion due to any micro-organism, is the development of a layer of compact, healthy granulations on the raw surfaces.

Bearing these facts in mind, one could not hope to show any startling effects produced by the employment of one procedure, or of any series of procedures; so that the results given herein must be critically interpreted.

The following questions naturally arise:

(1) Does the presence of anaërobes in wounds—the presence of anaërobes in general, not the presence of certain species of anaërobes—seriously interfere with the healing of a wound?

(2) Is there any particular method of treatment commonly in use in France which leads, either to elimination of anaërobic infection or to rapid healing?

(3) Is there any method in use in home hospitals which is especially useful in attaining these ends?

(4) Is there any one surgical procedure, apart from the dressings



used, which is followed by special rapidity of repair or by elimination of anaërobes?

The problem under investigation had therefore to be examined from such standpoints, that these questions might be answered as far as possible.

(c) *Does the presence of anaërobes in wounds seriously interfere with the process of healing?*

In dealing with a series of cases in which swabs were taken seven days before the wound had healed sufficiently for the patient to be considered convalescent, the following results were obtained:

- (a) No anaërobes were found in 19 instances.
- (b) Light anaërobe infection was present in 20 instances.
- (c) Heavy anaërobe infection was present in four instances.
- (d) Tetanus bacilli were demonstrated in the swabs in (c) in three instances.

Of 43 cases which failed to heal during the period over which the investigation extended—each wound was examined for five weeks at intervals of one week—the swab taken during the last week in which the patient was under examination yielded:

- (a) No anaërobes in 29 instances.
- (b) Light anaërobic infection in 14 instances.
- (c) Tetanus bacilli were present in one instance.

These figures indicate that anaërobic infection may persist in wounds up to the time of healing, and that the presence of such infection does not seriously retard the process of repair.

It must not be assumed from this statement, that an anaërobic infection is, in the opinion of the writers, of little or no importance in the pathology of the later phase of wound infection. The time, however, during which the presence of anaërobic bacilli is of special significance, is the first day or two after the injury has been received, and the variety, quite as much as the mass of the infection, is of importance at this time.

It must be appreciated too that, short of making a detailed inquiry into the flora of a large number of wounds, one cannot assume that the presence of anaërobes, even in the later phases of the process of repair, are of no significance; for the presence of bacilli of the *Vibrion septique* group, *B. Welchii* of *B. oedematiens*, and of *B. tetani* must always be a menace.

The detection however, of certain of these, is extremely difficult, and a special research would have to be devoted to the study of each, were a really satisfactory account to be given of the problem under consideration. Such detailed inquiry, in the present instance, has been therefore limited to the demonstration of *B. tetani* and of *B. Welchii*.

It is to be noted that in the case of *B. Welchii*, morphological characters were relied upon for demonstrating the presence of this bacillus—not a highly satisfactory method, but one which we were forced to adopt owing to the labour and expense which other methods would have involved.



(d) *Is there any method of treatment commonly used by the Army Surgeon abroad which tends to eliminate anaërobie infection?*

The wounds which were dealt with in the present series of examinations had, in the majority of instances, been treated either with flavine or with eusol, before removal to England. These wounds will therefore be grouped in three series:

- (a) Treated with flavine.
- (b) Treated with eusol or Carrel Dakin.
- (c) Treated by other methods.

In considering this question, it was decided to deal only with the wounds of men who arrived in England within ten weeks of the date of wounding. If such a period be not put to the type of case considered, the difficulty of assessing the value of any procedure employed is greatly increased, owing to the number of refractory cases which would be introduced into the series.

(a) *Cases treated with flavine before evacuation to England.*

Twenty-two cases of the present series were treated with flavine abroad, and arrived in England within ten weeks of the receipt of injury. From them 72 swabs were examined.

35 swabs showed no anaërobic infection

32 „ „ light „ „

5 „ „ heavy „ „

In none of these swabs was *B. tetani* found.

(b) *Cases treated with eusol or Carrel Dakin before evacuation to England.*

Of 78 swabs from 22 wounds treated with eusol abroad and sent to England within the ten-week period,

26 swabs failed to give growth of anaërobes

31 „ gave light „ „

21 „ „ heavy „ „

*B. tetani* appeared in eight swabs from six wounds in this series.

(c) *Cases treated by various methods.*

There remained 28 cases examined with the ten-week period comprising

10 treated with B.I.P.P.

7 „ dry dressings,

7 „ boric foment,

4 „ saline or salt packs.

I shall consider these together, as the number of each is too small to justify their being considered separately.

Ninety-two observations were made on the 28 wounds, and the following results were obtained:

42 showed no growth of anaërobes,

39 „ light „ „

11 „ heavy „ „

*B. tetani* was grown from seven swabs obtained from five different wounds. Diagram VII shows these results expressed as percentages.

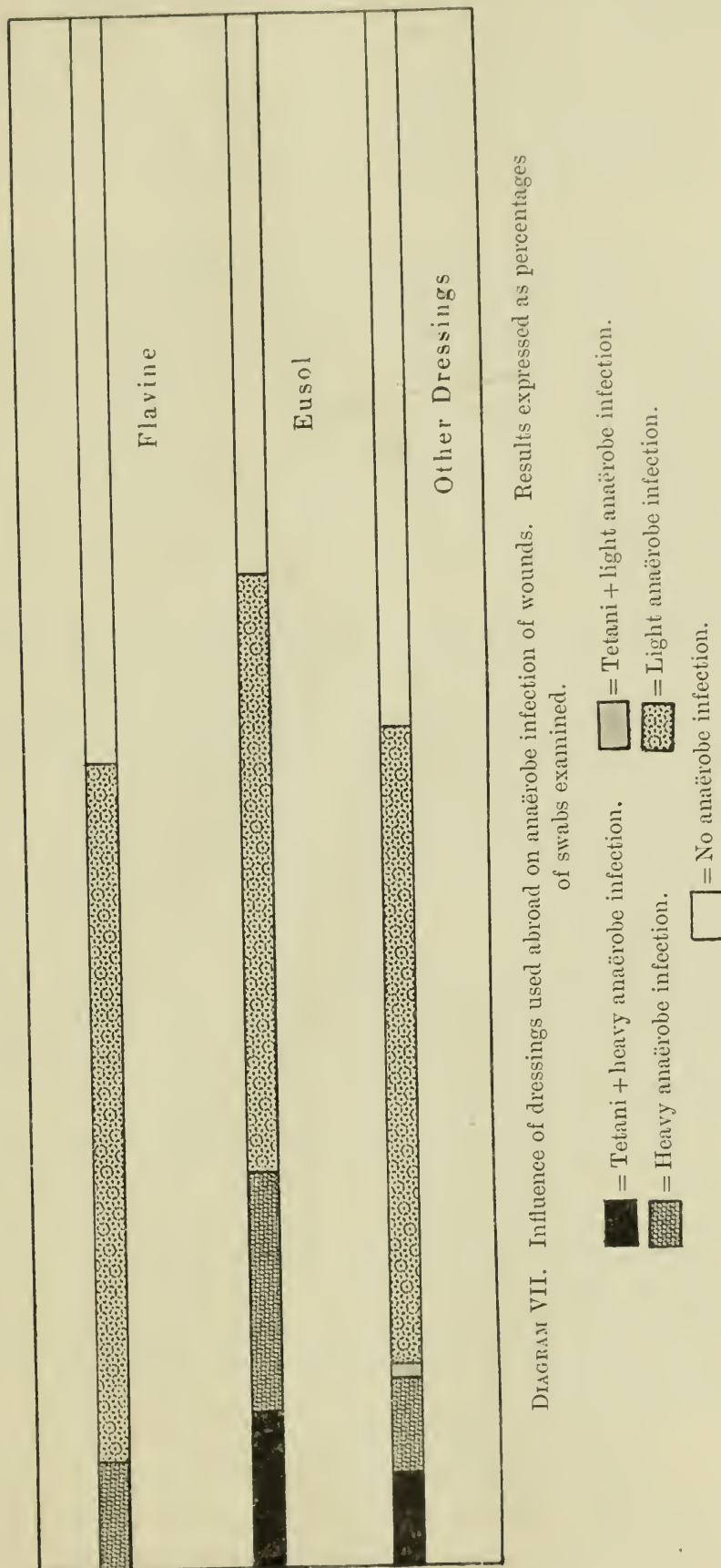


DIAGRAM VII. Influence of dressings used abroad on anaërobe infection of wounds. Results expressed as percentages of swabs examined.

The dark columns refer to heavy anaërobe infection, the light to light anaërobe infection. The black columns indicate that *B. tetani* was found along with heavy infections of other anaërobes, and the hatched column that *B. tetani* was present with light concomitant anaërobe infection.

It is not easy to comment on the results shown in the above diagram, for they may well give a false impression, owing to the fact that 11 of the wounds in the flavine series had been excised, while only six of those of the eusol series, and five of those in which other dressings had been used, were so treated.

Both the low rate of "heavy anaërobe infection" and the absence of tetanus bacilli in those wounds originally treated by flavine, appear to suggest that that reagent is suitable for primary dressings. The results may, however, mean that excision of the wounds was, in the instances quoted, the most important factor in eliminating infection.

This subject will be again considered in dealing with the "time factor" in the healing of excised wounds, as compared with that of non-excised wounds.

In Diagrams XIII and XIV (pp. 191, 192) are graphically shown the results of each examination of the swabs dealt with above.

(e) *Influence of dressings in use in the Home Hospitals on the degree and persistence of anaërobic infection of wounds.*

In this series, the arbitrary time limit of ten weeks was again adhered to, in order that the results obtained might be, as far as possible, comparable with those dealing with the methods used abroad.

The series comprises an additional three cases in which the treatment abroad was not recorded on the field cards, so that 75 in place of 72 cases are herein considered.

(a) *Cases treated by boric fomentations.*

It was surprising to find that boric fomentation was the favourite method of treatment in the hospitals from which the material was obtained, and of cases so treated there were 35 involving 123 observations.

55 swabs failed to give growth of anaërobes,

49   ,,   gave light       ,,       ,,

19   ,,       ,,   heavy       ,,       ,,

*B. tetani* was obtained from 11 swabs in seven cases.

(b) *Cases treated by eusol.*

Of cases treated by eusol or the Carrel Dakin method there are 22, involving 58 observations.

27 showed no anaërobe infection,

23   ,,   light       ,,       ,,

8    ,,   heavy   ,,       ,,

*B. tetani* was obtained from three swabs in three cases.



*(c) Cases treated by other methods.*

Eighteen cases of the series were treated by other methods (comprising 13 in which saline was used), in which a variety of procedures had been employed.

These involved 71 observations, giving the following results.

30	swabs failed to give growth of anaërobes,
30	„ showed light anaërobe infection,
11	„ heavy „ „

*B. tetani* was found in three instances from three cases.

These results are summarised in Diagram VIII, p. 180.

These results are instructive, for they show in a remarkable manner, that no particular dressing can be recommended as likely to produce marked diminution in the anaërobe flora of wounds during the process of repair.

Experimental evidence bears this out—thus, Brilliant Green, which in certain low concentrations will markedly inhibit the growth of bacteria, including the anaërobes, in broth or in serum, is much reduced in its inhibitory activity if a piece of fresh living tissue be added to the cultures.

The conditions obtaining in cultures in fluid media enriched by the addition of fresh tissue, more closely approximate the conditions of a wound, than do cultures which are not so enriched.

It is probable that this inhibition of antiseptic activity demonstrated in culture tubes is multiplied manifold in wounds, owing to the large surface of granulation to which the reagent is exposed.

All that can be hoped for then, from the use of any antiseptic so far employed, is, that it will reduce mass infection of wound exudates; it will not, however, eliminate infection.

As a mild degree of anaërobe infection is quite compatible, so far as can be seen, with unretarded progress of healing; and, as we cannot hope to eliminate the anaërobes by the use of any antiseptic, the question at issue must be dealt with from another point of view.

The details of the examination of each swab are given in Diagrams XV and XVI (pp. 193, 194) at the end of this section.

*(f) Rapidity with which healing takes place under various dressings.*

So long as a wound remains open it is, to a greater or less degree, susceptible to bacterial invasion of all kinds. If then, any particular procedure can be shown to cause rapid healing of wounds, such is to be recommended, both from the standpoint of surgery and from that of economics.

Three points of view have to be considered in dealing with this question.

(a) The nature of the dressing used abroad.

(b) The nature of the dressing in home hospitals.

(c) The wounds treated by excision must be contrasted with those that have not been excised.

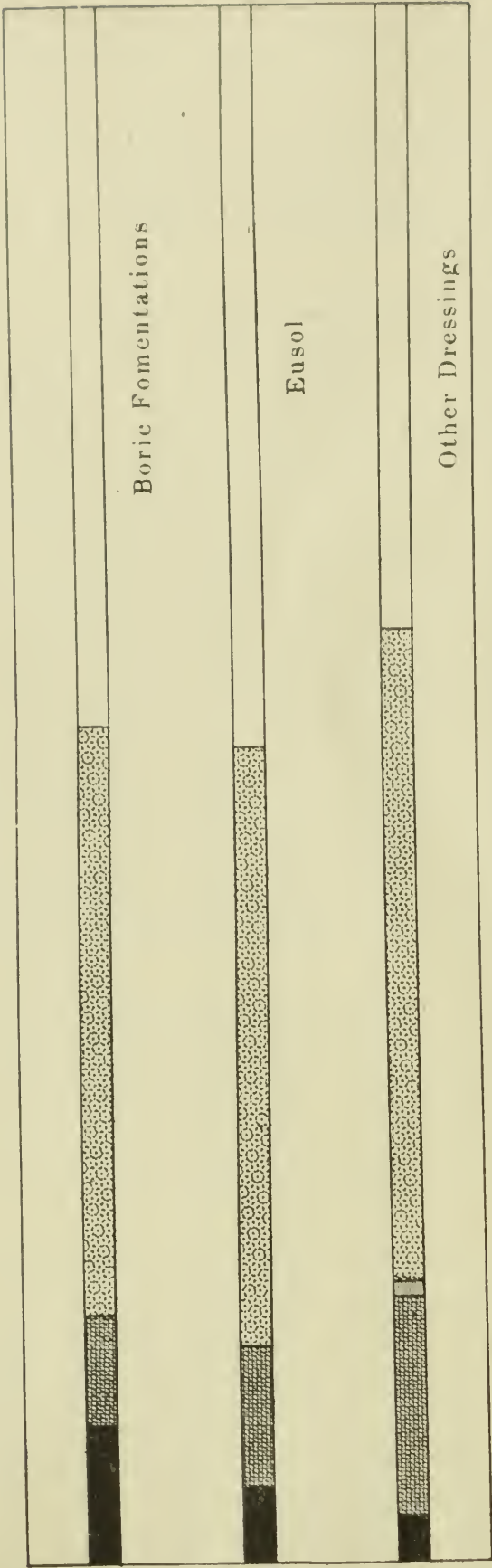







DIAGRAM VIII. Influence of dressings used in Home Hospitals on anaërobe infection. Results expressed as percentages of swabs examined.

 = Tetani + heavy anaërobe infection.  = Tetani + light anaërobe infection.

 = Heavy anaërobe infection.  = Light anaërobe infection.

 = No anaërobe infection.

*(f') Influence of dressings used abroad upon the rate of healing.*1. *Flavine.*

Twenty-one wounds fulfilling the conditions arbitrarily laid down concerning evacuation to England within a ten-week period, were treated with flavine abroad. Of these 14 recovered, the recoveries being distributed thus:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	2	2
2 weeks	8	6
3 "	4	4
4 "	2	0
5 "	1	0
6 "	1	0
7 "	1	0
8 "	0	0
9 "	2	2
10 "	0	0

2. *Eusol.*

Twenty-two wounds receiving early treatment with this reagent behaved as follows:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	3	2
2 weeks	3	1
3 "	3	1
4 "	2	1
5 "	3	2
6 "	2	0
7 "	1	0
8 "	1	1
9 "	1	0
10 "	3	2

3. *Other methods.*

In the case of 26 wounds which had been treated abroad by other methods, the following results were obtained:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	4	3
2 weeks	8	5
3 "	5	1
4 "	2	2
5 "	0	0
6 "	1	0
7 "	2	0
8 "	2	2
9 "	0	0
10 "	2	0



These results are summarised in Diagram IX, in which both the recovery rate and the time when recovery occurred are graphically shown. For the purposes of comparison the figures are reduced to a common denominator.

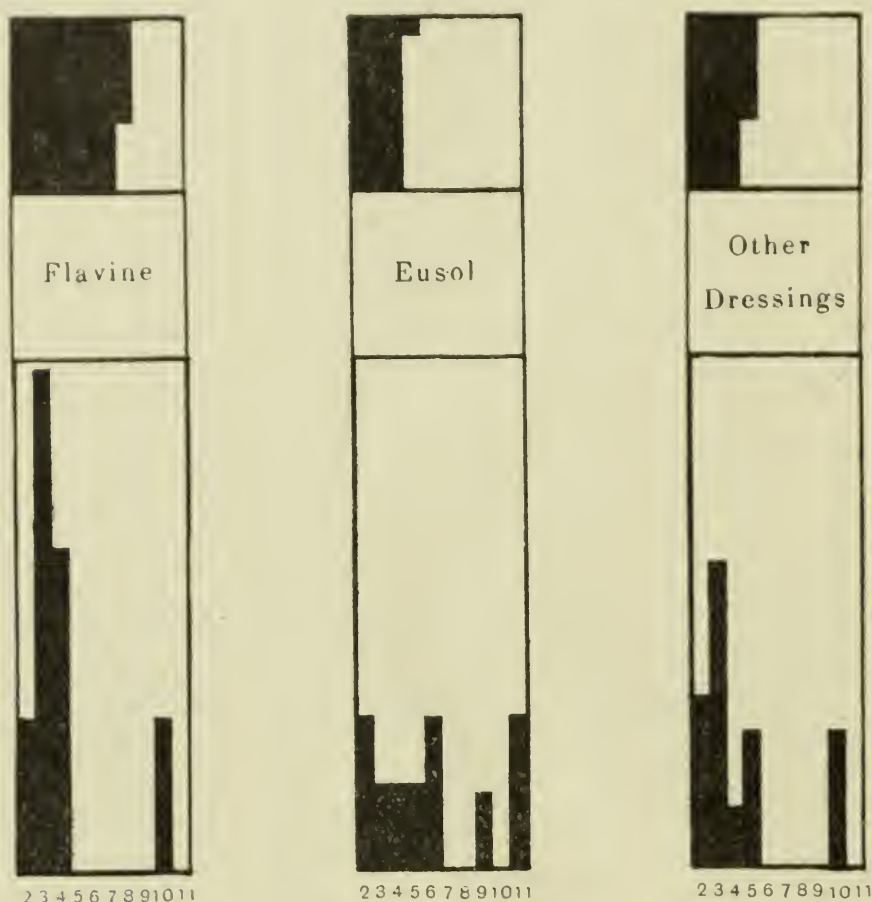


DIAGRAM IX. The upper portion of the diagram indicates the percentage recovery with each dressing. The lower indicates the wounds which recovered. Figures relate to week in which recovered cases were first examined in England. An arbitrary time limit of five weeks from first examination was taken as period for recovery to take place.

These results appear to indicate that flavine is the most valuable dressing of those considered in the present series, both as regards the number of recoveries and the rapidity of recovery.

It must be noted, however, that there is a preponderance of excised wounds in the flavine series.

#### *(f'') Influence of dressings used in Home Hospitals.*

When considered from the standpoint of dressings used in Home Hospitals, the following results appear:

##### *1. Boric fomentations.*

Thirty-six wounds were treated by boric fomentations, with 12 recoveries, distributed as follows:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	5	2
2 weeks	7	4
3 ..	7	3
4 ..	3	1
5 ..	3	0
6 ..	2	0
7 ..	3	0
8 ..	0	0
9 ..	6	2
10 ..	0	0

### 2. *Eusol*.

Twenty-two wounds were treated with eusol, giving 15 recoveries, distributed as follows:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	4	4
2 weeks	5	4
3 ..	3	2
4 ..	1	1
5 ..	2	1
6 ..	1	1
7 ..	4	2
8 ..	0	0
9 ..	0	0
10 ..	1	0

### 3. *Other methods*.

Eighteen wounds were treated by other methods—with nine recoveries, distributed as follows:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	0	0
2 weeks	5	4
3 ..	8	2
4 ..	2	1
5 ..	1	1
6 ..	0	0
7 ..	1	1
8 ..	0	0
9 ..	0	0
10 ..	1	0

*Note.* Of the series treated by boric fomentations four had been excised. Of those treated with eusol nine and of those by other methods three.

These results are summarised in Diagram X, p. 184, which shows graphically both the recovery rate and the time when recovery took place.

The results summarised in Diagram X suggest that the cases treated with eusol in Home Hospitals heal more rapidly than do those treated by other methods. Here again, however, there is seen a preponderance of the excised wounds in the series.

The results then do not give information as to which dressings of those examined could be recommended for use in England, as being of special value in stimulating healing.

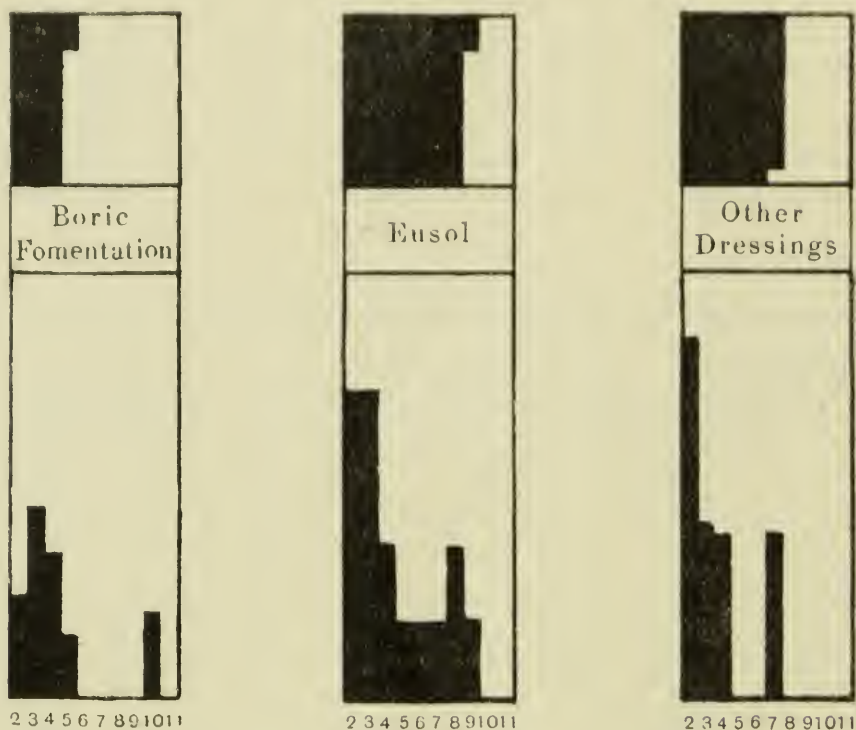


DIAGRAM X. The upper portion of the diagram indicates the percentage recovery with each dressing. The lower indicates the wounds which recovered. Figures relate to week in which recovered cases were first examined in England. An arbitrary time limit of five weeks from first examination was taken as period for recovery to take place.

(g) Influence of excision.

*Is there any one surgical procedure, apart from the dressings employed, which leads to the rapid healing of wounds?*

If excised wounds be contrasted with those not so treated, it is seen that within the arbitrary period of ten weeks, 16 out of 26 excised wounds had progressed sufficiently for the patients to be considered convalescent. While of the non-excised wounds, 51 in number, 24 became convalescent. These findings are summarised in Diagram XI, in which are indicated both the recovery rate, and the time within which recovery occurred.

This diagram indicates:

- (a) That the recovery rate is higher in the *excised* than in the *non-excised* wounds.
- (b) That the distribution of the recoveries in point of time is more uniform in the *excised* than in the *non-excised* wounds. This latter point appears to the writers to be a matter of some importance, as it suggests, that a more diverse type of wound heals within a given period after excision, than is the case when excision is not practised. This really means, that in the



case of the *non-excised* wounds, unless healing occurs within the first three or four weeks, the convalescence may be protracted.

The details of the examination of swabs from *excised wounds*, irrespective of the dressing used, are shown graphically in Diagram XVII, p. 195.

Before proceeding to the next sub-section of the investigation, dealing with wounds, the examination of which was commenced after the expiry of

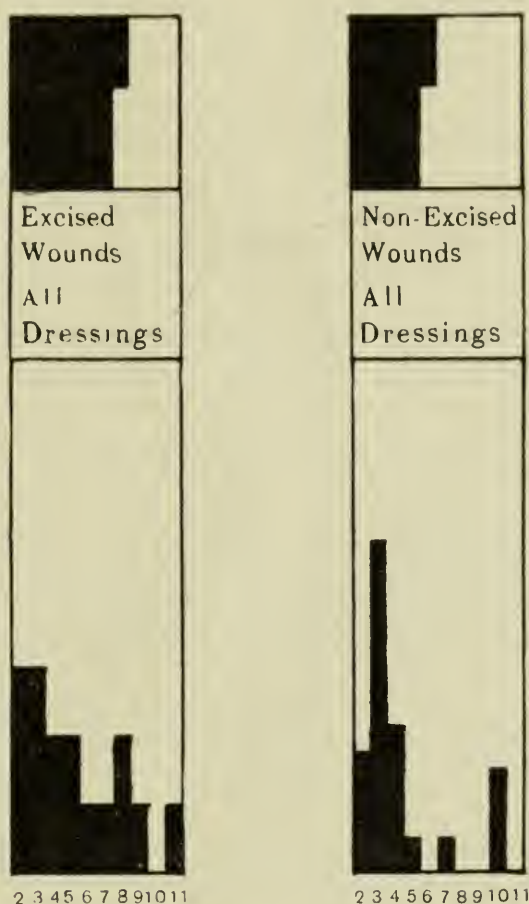


DIAGRAM XI. The upper portion of the diagram indicates the percentage recovery in each instant. The lower indicates those wounds which recovered. Figures relate to week in which recovered cases were first examined in England. An arbitrary time limit of five weeks from first examination was taken as period for recovery to take place.

the ten-week period, the question of the influence which anaërobes have upon healing, and also the influence which various dressings exert upon anaërobe infection, may be summarised thus:

(i) A mild degree of anaërobe infection does not prevent, and does not apparently even retard, the process of healing.

(ii) Wounds treated with flavine in France have, on the whole, a lesser degree of infection with anaërobes, than those treated by other methods. The fallacy due to the preponderance of *excised* wounds so treated, renders it difficult to offer an opinion as to whether flavine or excision has been the factor producing the desired result.

(iii) None of the treatments used in home hospitals, which we were in a position to investigate, eliminate anaërobe infection with especial rapidity.

(iv) As regards the rapidity with which the wounds healed, the best results were obtained in those treated with flavine abroad and with eusol in England. It is to be noted that a preponderance of *excised* wounds in both instances renders difficult the assessment of these results.

(v) On contrasting *excised* with *non-excised* wounds, it is found, that the recovery rate is higher and more uniform in wounds which have been *excised* than in those which have not been so treated.

(h) *Examination of wounds of long standing.*

In addition to the series that has already been considered, 12 wounds were examined in men who arrived in England between the 10th and 15th weeks after reception of the injury. This involved the examination of 37 swabs.

No anaërobes found in 23 instances,

Light anaërobic infection in 10 instances,

Heavy       ,,               ,,               4       ,,

*B. tetani* was found in seven swabs from five cases.

Finally, as a matter of scientific interest, it was decided to examine some wounds of very long standing.

(a) Wound examined during the 17th and 18th weeks gave one swab which showed a heavy growth, and one which failed to give growth of anaërobes.

(b) Wound examined from the 18th to the 23rd week gave three swabs producing a heavy growth of anaërobes, and two which showed no growth of such organisms.

(c) Wound examined from the 22nd to the 25th week gave two swabs showing heavy anaërobic infection, one with light infection, while in two anaërobes failed to grow.

(d) Wound examined from the 23rd to the 27th week gave three swabs with light anaërobe infection and two with none.

(e) Wound examined from the 30th to the 33rd week gave no growth of anaërobes.

(f) Wound examined during the 32nd and 33rd weeks gave one swab with a light anaërobe infection and one showed no anaërobe infection.

(g) Wound examined from the 35th to the 38th week, one swab showed heavy growth of anaërobes and three showed no growth of anaërobes.

(h) Wound examined from the 37th to the 40th week all four swabs failed to give growth of anaërobes.

(i) Wound examined from the 38th to the 41st week gave four successive swabs showing heavy anaërobe infection.

(j) Wound examined from the 42nd to the 45th week gave three swabs showing light anaërobe infection and one showing none.



(k) Wound examined from the 43rd to the 47th week gave five successive swabs containing *B. tetani*, three being accompanied by heavy and two with light anaërobe infection.

(l) Wound examined from the 44th to the 47th week gave one swab with light anaërobe infection and three with none.

If then the results obtained in examining wounds between the 15th and 50th weeks after receipt of injury be summarised, the following results are obtained:

24 wounds were examined involving the examination of 82 swabs.

In 46 instances no anaërobes were found,

„ 22 „ light anaërobe infection was found,

„ 14 „ heavy „ „ „

*B. tetani* was obtained in 12 swabs from six cases.

On six occasions *B. tetani* was found along with heavy anaërobe infection and on the other occasions with light anaërobe infection.

It seems at first sight remarkable that these results are so bad, and compare unfavourably with any that have gone before, but the wounds considered are practically picked refractory cases and therefore occupy a category by themselves.

There are, in addition, two especially interesting observations:

(i) Five swabs were taken from a case between 104th and 108th weeks from date of wounding, three out of the five swabs were heavily infected with anaërobes, two out of the three containing *B. tetani*.

(ii) Five swabs were also taken from a case between the 106th and 130th weeks after reception of the injury, two gave a heavy growth of anaërobes *B. tetani* being present in large numbers in the cultures, one gave a light growth of anaërobes, and two gave no growth of these organisms.

These observations call attention to the remarkably long periods during which *B. tetani* and other anaërobes may persist in wounds. The importance of the observations is, that they indicate how necessary it is to administer a prophylactic dose of antitoxin the day before any operation for cosmetic or other purpose is performed at the site of an old wound. It would be advisable, too, to give, if possible, anti-gas-gangrene serum along with the prophylactic tetanus antitoxic serum.

(i) *Examination for presence of B. tetani in a series of 100 wounds.*

The tetanus bacilli obtained from 100 consecutive wounds of men showing no evidence of tetanus are tabulated on p. 188.

Commenting on these results, it is seen, that 14 cultures elaborating spasm-producing toxin were obtained from 100 consecutive wounds. In carrying out the toxin tests 0.2 c.c. was the largest quantity employed, when mice were the experimental animals used.

In four instances, while the toxin experiments were negative, typical



No.	Animal experiment	Agglutina- tion	Since wounding	Where wounded
1.	Toxin experiment positive	Type I	7 days	Ypres
2.	" " "	"	7 "	Ypres
3.	" " "	"	22 "	Ypres
4.	" " negative Infection experiment positive	"	24 "	Albert
5.	Toxin experiment "	"	25 "	Villers Bretonneux
6.	" " "	"	30 "	Mericourt
7.	" " negative Infection experiment positive	"	32 "	Cambrai
8.	Toxin experiment negative Infection experiment positive	"	35 "	Cambrai
9.	Toxin experiment negative Infection experiment positive	"	39 "	Cambrai
10.	Toxin experiment "	"	64 "	Battersea
11.	" " "	"	112 "	Ypres
12.	" " "	"	130 "	Cambrai
13.	See Footnote 1	"	733 "	Hohenzollern Re- doubt
14.	Toxin experiment positive	"	860 "	Poelcapelle
15.	" " "	"	882 "	Cambrai
16.	" " "	Type II	15 "	Bapaume
17.	" " "	"	138 "	Passchendaele
18.	See Footnote 2	"	100 "	Cambrai
19.	" "	"	54 "	Monchy
20.	Toxin experiment positive	Type III	16 "	Orvillers
21.	" " "	Type IV	305 "	Hermies

*Footnote 1.* With reference to No. 13 toxin experiments were negative, and when an attempt was made to carry out the infection experiment, the animals died from gas gangrene.

*Footnote 2.* Cultures 18 and 19. Culture 18, which agglutinated in presence of Type II serum, was lost before the animal experiments were completed. Culture 19 is still under observation.

tetanus infection could be produced when washed cultures were injected together with a tissue debilitant. Therefore, in at least 18 per cent. of the present series of wounds, tetanus bacilli could be recovered.

This figure is considerably higher than any previous investigations would have led one to expect. The reason for this somewhat high figure is, that unless wounds be repeatedly examined, tetanus bacilli if present in small numbers may be missed; just as throat swabs, in cases of diphtheria, may give negative results owing to the operator having failed to swab that area of the throat in which *B. diphtheriae* is present.

Diagrams XIII, XIV, XV, XVI and XVII which give the result of the examination of each swab graphically illustrate this point.

(j) *Examination for presence of organisms having the morphological characters of B. Welchii in a series of 100 wounds.*

In the series of wounds under consideration the presence of organisms having the morphological appearance of *B. Welchii* was also noted, and an attempt was made to determine whether any of the dressings commonly

used, either at home or abroad, tended rapidly to eliminate this organism from wound exudates.

From this inquiry, the following conclusion was drawn: "That no dressing among those investigated could be especially recommended as likely to produce rapid elimination of *B. Welchii*."

As the details of the examination constitute what is virtually a repetition of the findings already set forth in connection with the inquiry into the influence which various dressings exert upon the reduction of all anaërobic infections, they will not be dealt with *in extenso*.

The results of the examinations call attention, however, to a marked difference between *excised* and *non-excised* wounds in this connection. While there is but little difference between the percentages of *excised* and *non-excised* wounds, the exudates of which contain *B. Welchii*, there is a marked difference between the two classes in respect of the period over which infection with *B. Welchii* can be demonstrated. This period is much shorter in the case of *excised* wounds than in the case of *non-excised* wounds.

The actual findings are as follows:

On a series of 100 wounds 46 were found to contain *B. Welchii*.

(a) In four wounds—two excised, two non-excised—*B. Welchii* disappeared before the second week.

(b) In eight wounds—five excised, three non-excised—*B. Welchii* disappeared before the third week.

(c) In six wounds—one excised, five non-excised—*B. Welchii* disappeared before the fourth week.

(d) In three non-excised wounds, it disappeared before the fifth week.

(e) In four wounds—three excised, one non-excised—it disappeared before the sixth week.

(f) In two wounds—one excised, one non-excised—it disappeared before the seventh week.

(g) Thereafter, only non-excised wounds contained *B. Welchii*. In one this organism disappeared before the 8th week, three before the 9th week, one before the 11th, four before the 13th, one before the 15th, one before the 16th, two before the 17th; and five wounds were shown to contain *B. Welchii* between the 27th and 47th weeks after infliction of the injury. These facts are set forth diagrammatically in the following figure.

*Note.* Attention is called to the fact, that in many of the instances quoted above, the number of organisms present in the cultures which had the morphological appearance of *B. Welchii* was small. It is probable that, without careful and repeated examination of the growths, the presence of this bacillus would not have been appreciated.

These findings are in full agreement with those which were obtained in making inquiry into the influence which various dressings and surgical procedures exert upon all anaërobic infections of wounds.

They are especially valuable, in that they show fairly conclusively, “that excision does not eliminate infection with the anaërobic bacilli but it removes

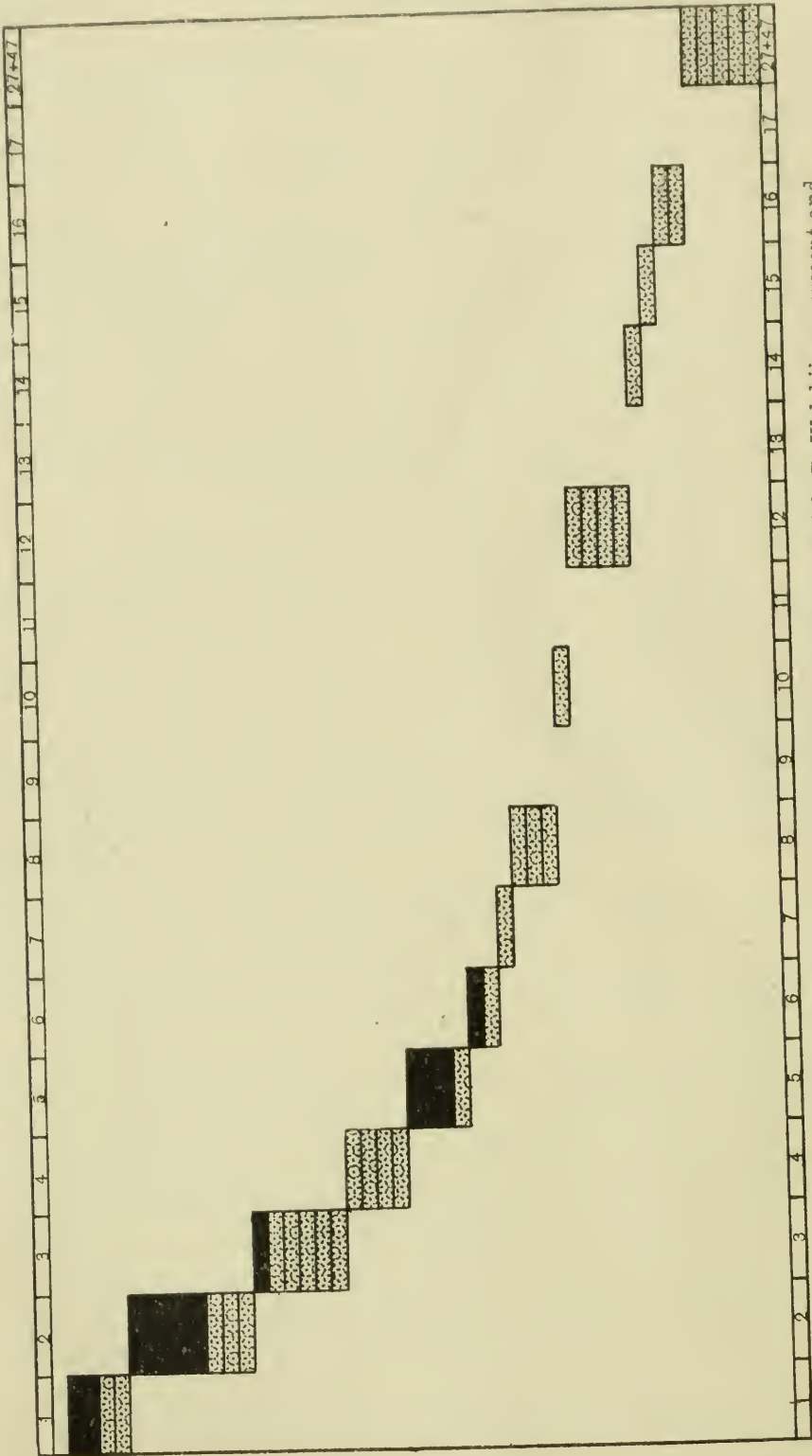


DIAGRAM XII. This figure shows the number of wounds in which *B. Welchii* was present and indicates the last swab in which it could be demonstrated in each instance.

■ = In excised wounds.  
▨ = In non-excised wounds.  
Figures = Weeks since reception of injury.  
Total number of excised wounds = 27, *Welchii* present in 12 = 44 %.  
Total number of non-excised wounds = 73, *Welchii* present in 34 = 46 %.

those conditions which enhance the danger arising from the presence of these organisms.”



DIAGRAM XIII.

Results of Weekly Examination  
of Wounds Treated Abroad with  
Flavine and with Eusol

■ = Tetani + Heavy Anaerobe Infection  
▨ = Heavy Anaerobe Infection  
▩ = Light Anaerobe Infection

C = Recovery

E = Excised Wound

Figures = Weeks since Reception of Injury

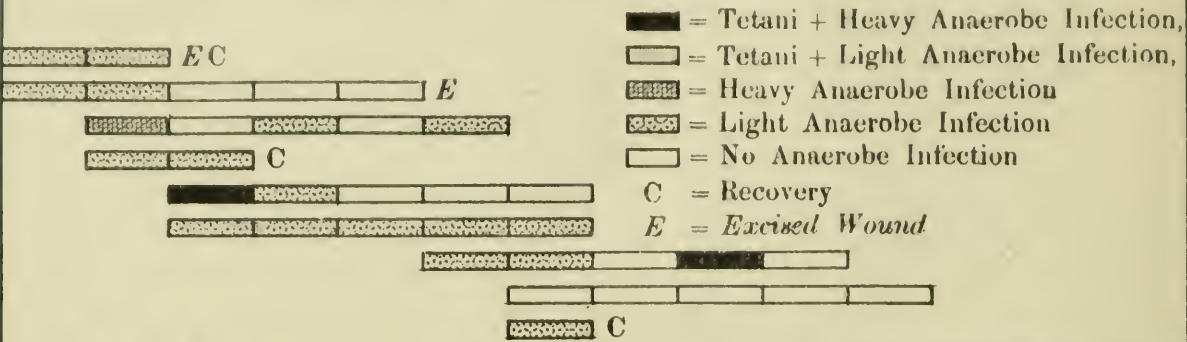
Flavine

C

Eusol

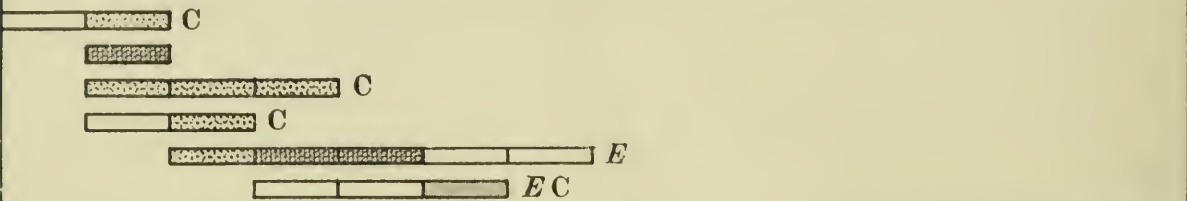
C  
EC

DIAGRAM XIV. Results of Weekly Examinations of Wounds Treated Abroad with B.I.P.P., Dry Dressings, Boric Foments, and Saline

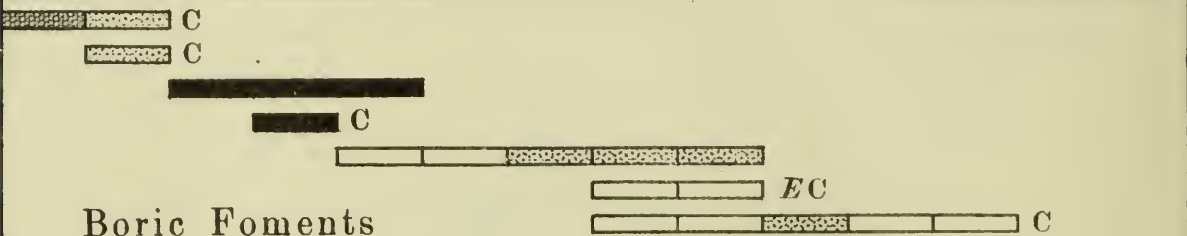


B.I.P.P.

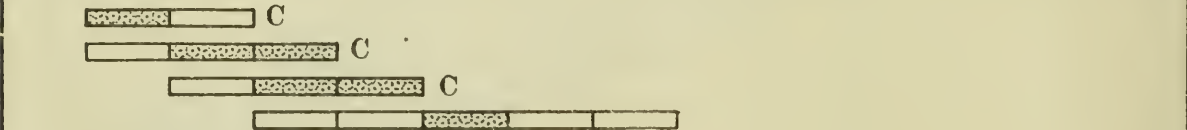
Figures = Weeks since Reception of Injury



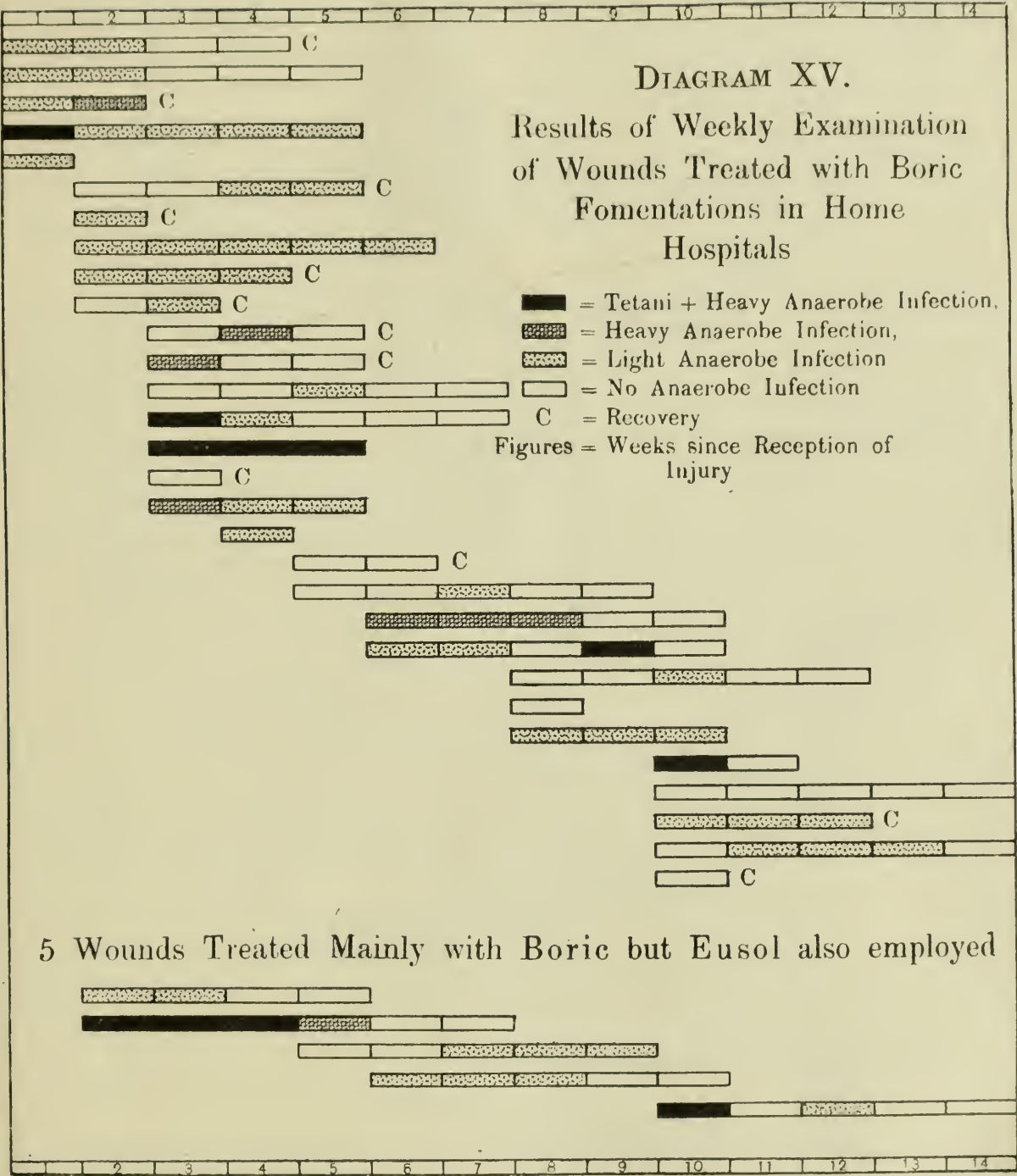
Dry Dressings



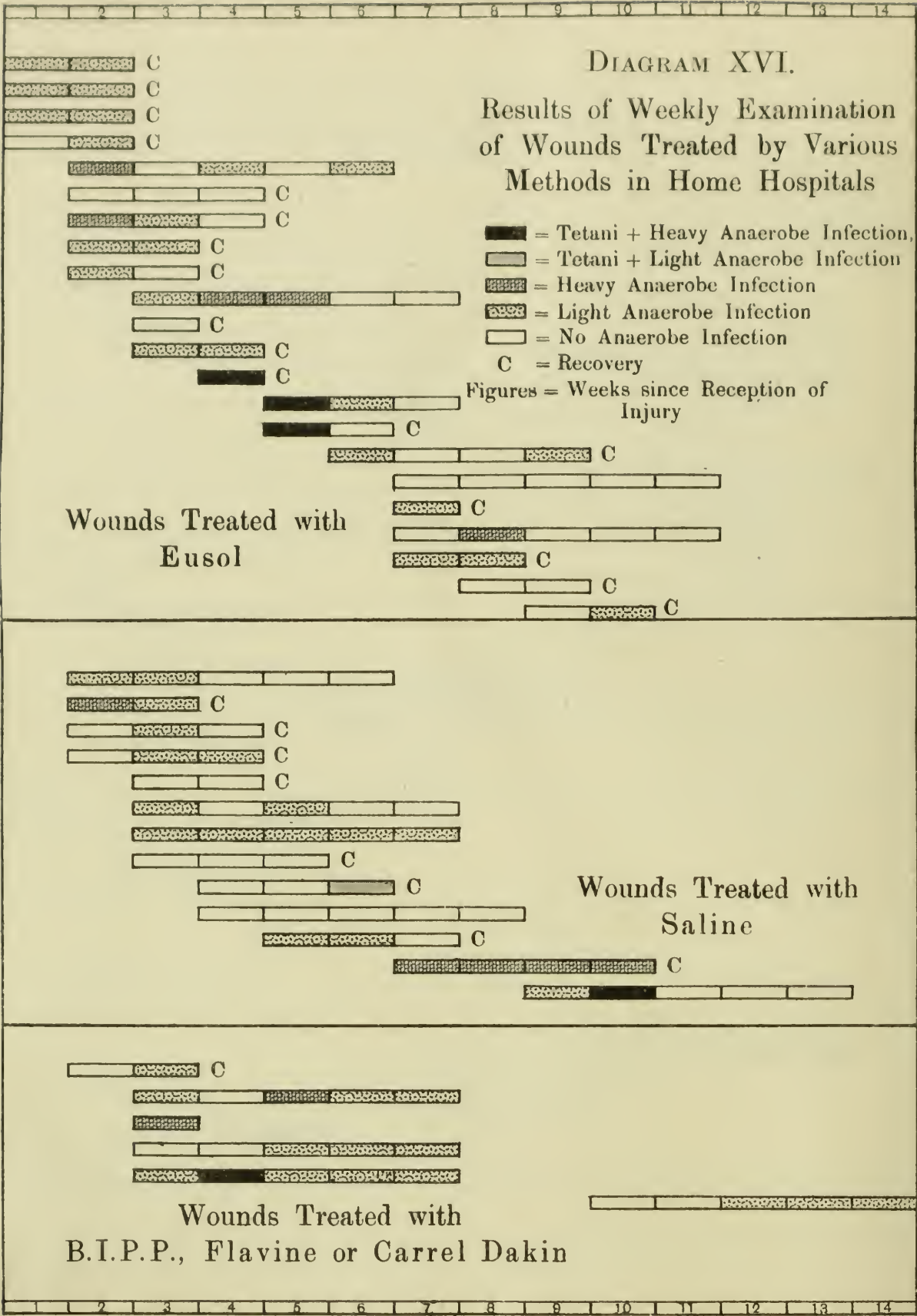
Boric Foments



Saline or Salt Pack

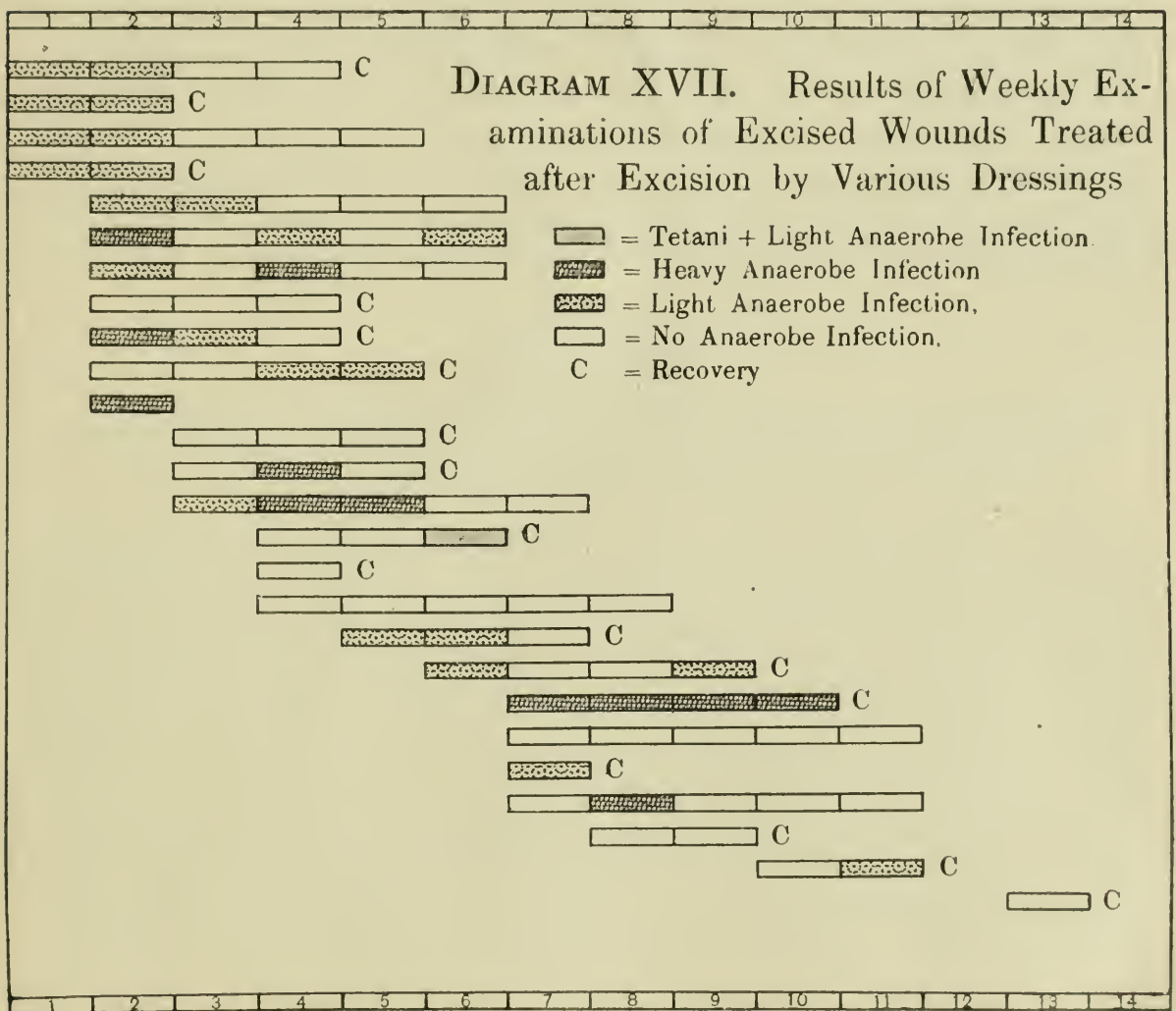






CONCLUSIONS FROM SECTION VI, pp. 172-195.

1. No one dressing, of those dealt with in the present investigation, can be especially recommended as being valuable for the elimination of anaërobic micro-organisms in general from wound exudates.
2. Neither can any one dressing be recommended as especially valuable in eliminating *B. tetani* or *B. Welchii*.
3. The presence of a mild degree of anaërobe infection in wounds does not necessarily prevent, or even retard, the process of healing.
4. Excised wounds heal more rapidly than do non-excised wounds, and therefore by excision the danger period is reduced during which infection with either *B. tetani* or the gas gangrene bacilli may be a menace.



## SECTION VII.

CAN AGGLUTINATION OF STOCK EMULSIONS OF *B. TETANI* BY THE SERUM OF PATIENTS SUFFERING FROM EARLY TETANUS BE EMPLOYED AS A METHOD FOR DIAGNOSIS AND FOR DETERMINING "TYPE OF INFECTION" IN TETANUS?

Serum from over 60 cases of tetanus was used for agglutinating stock emulsions of the 4 Type bacilli. At the same time a number of normal sera were tested in the same way.

The result showed that certain normal sera may agglutinate tetanus bacilli of one or other Type in dilutions up to 1/80. None of the sera from cases of the disease could be definitely demonstrated to be of much higher titre; and further, in those cases in which the infecting organism was typed, no relationship could be shown between the Type of the infecting organism and the agglutination reaction performed with the patient's serum. One had to conclude, therefore, that agglutination could not be employed for making a "preclinical" diagnosis of tetanus, nor could it be used for determining to which serological Type of the bacillus the organism belonged which was responsible for the causation of the disease in each case.

## SECTION VIII.

RELATION OF SEROLOGICAL TYPE OF BACILLUS ISOLATED, TO HAEMAGGLUTINATING TYPE OF MEN FROM WHOM THE BACILLUS WAS OBTAINED.

As a matter of scientific interest, the haemagglutinating property of these sera was, where possible, determined. The reason for undertaking this inquiry was, that it might be suggested, that the serological Type of the organism depended upon its being parasitic on individuals belonging to a particular haemagglutinating group of men; for instance, the Type I bacillus might evolve as a result of its being parasitic upon an individual of haemagglutinin Group II. Type III bacillus might result from parasitism on an individual belonging to another haemagglutinating group, etc. Such a relationship could not be demonstrated; for example, of four individuals from whom Type III bacilli were isolated, two belonged to haemagglutinin Group II, one to Group III, and one to Group IV.

## SECTION IX.

A NOTE ON TWO CASES OF ABDOMINAL TETANUS.

The two following cases are of especial interest, in that tetanus developed after the performance of an abdominal operation, and the source of infection in each case was undoubtedly the intestine.



*Case I.*

A man, who had been wounded in France one year previously, was taken ill with appendicitis; some days after the operation tetanus developed with a fatal result.

Examination of material from the wound in the abdominal wall, from the stump of the appendix, and from the faeces in the descending colon, all yielded a growth of tetanus bacilli of serological Type I and all virulent for animals.

*Case II.*

In this case, which was forwarded to me unofficially, the victim of the disease was a girl of 4½. The history of the case was as follows:

Patient was admitted suffering from intussusception—a laparotomy was performed the same day and the intussusception was reduced. Five days later tetanus developed and terminated fatally. Material from the wound and faeces were both submitted for examination. Type III tetanus bacilli were found in both.

The main interest of the second case is that as the gut had not been opened, therefore the bacillus must have been in the gut, and the cause of the *infection* was probably the tissue debility produced by the intussusception.

## SECTION X.

PRESENCE OF *B. TETANI* IN THE FAECES OF MEN RETURNED FROM OVERSEAS COMPARED WITH THOSE FROM CIVILIAN FAECES.

Up to the present 21 specimens of faeces from men returned from overseas have been examined, and in seven instances organisms were obtained which agglutinated with one or other of the Type agglutinating sera. Of these, four were Type I bacilli, two were Type II and one was Type III. Of 31 specimens of faeces from civilians five gave cultures which agglutinated with Type sera. All proved to be Type I. It is to be noted that the investigation of these faeces was not so complete as was that of cultures obtained either from the wounds of men suffering from tetanus or from wounds of men showing no clinical evidence of tetanus.

The inquiry, therefore, makes no claim to accuracy, and the results obtained are mainly of academic interest.

The reason for conducting this inquiry was, that a number of cases of abdominal tetanus in soldiers had been reported to Major-General Sir David Bruce, K.C.B., F.R.S. The aggregate of these cases was considerable. Two explanations of their occurrence had to be considered:

(i) It might be that owing to the machinery for collecting information *re* tetanus being adequate, these cases were returned among those due to tetanus infection of war wounds. Seen together the number appeared to be

relatively large, but without consulting the Registrar General's returns, it could not be assumed that the number was larger than in times of peace.

(ii) It might be, on the other hand, that the conditions of active service leading to contamination of food stuffs, etc., with earth, resulted in sufficient unconscious geophagy to alter the flora of the gut, and to increase the number of bacillus tetani. The figures quoted are insufficient to demonstrate that the latter explanation is correct, and it is highly probable that the larger number of cases of abdominal tetanus among soldiers is apparent rather than real.

Nevertheless in view of the findings reported in this section, and in view also of the findings previously published by Pizzini, it might be advisable to err on the side of safety, and administer a prophylactic dose of antitoxin before performing an abdominal operation involving section of the gut, especially when such operations are performed on recently returned men.

### SYNOPSIS AND GENERAL CONCLUSIONS.

I. In Sections I, II, and III, are described the methods employed, and the results obtained, in making a systematic investigation of the prevalence of the various serological Types of *B. tetani*, both in wounds of men suffering from tetanus and in those of men showing no evidence of that disease.

This inquiry shows:

1. That the mortality among inoculated men from infection due to tetanus bacilli belonging to serological Type I is lower than that from infection with either Type II or Type III.

2. If the observations be limited to those cases in which the incubation period is 14 days or less, the mortality from infection due to Types II and III is higher than when Type I is the organism responsible for the causation of the disease.

3. If observations on the incidence of cases due to the various Types of *B. tetani* be considered from the same point of view, relatively more cases of Type II and Type III infection declare themselves within a period of 14 days than Type I.

4. From an unexpectedly large percentage—20 per cent.—of wounds of men who show no evidence of tetanus, *B. tetani* can be recovered at some period during the process of repair.

5. Of tetanus bacilli obtained from such "indifferent" wounds the majority conform to serological Type I.

These facts are susceptible of two explanations:

A. Either that Type I bacilli are, on the whole, less virulent, or less toxogenic, in character than are those of Types II and III;

B. Or, that the serum prophylaxis in use until recently, afforded more adequate protection against Type I infections than it did against infections due to Types II or III.



As all those cultures of *B. tetani* that I obtained from serum laboratories conformed to Type I on serological examination, it appeared that hypothesis B was worthy of serious consideration.

II. Section IV deals with experiments that were undertaken to demonstrate *in vitro* the stability of the Types after prolonged culture, and to investigate the presence of antibodies other than agglutinins in the sera of animals immunised by inoculating

- (a) the filtered products of growth—"Toxin,"
- (b) washed bacilli,
- (c) whole cultures.

The facts obtained from this section of the inquiry are:

1. That even when agglutinating sera of high titre are used the bacillary Types react specifically.
2. That even after very frequent sub-culture extending over a period exceeding one year, the bacilli remain true to type.
3. Anti-bacterial qualities, other than agglutinins, can be evoked by inoculating whole cultures of *B. tetani* into animals. For technical reasons the anti-bacterial body which was most fully studied was of the nature of a "stimulin" or "opsonin."
4. Antitoxic serum obtained by inoculation of culture filtrates does not stimulate phagocytosis of whole culture.
5. Inoculation of *washed* cultures does not evoke the elaboration of so active an "opsonin" (for *whole* cultures) as does inoculation of *whole* cultures.
6. *Washed* culture and *whole* culture appear to be equally active in evoking agglutinin production.

The following deductions therefore seem permissible: that in *whole* cultures of *B. tetani* three antigens, at least, appear to exist.

(a) The *bacillary* substance itself—the inoculation of this leads to the development of agglutinins which are specific to the Types.

(b) An *antigen*, which is anti-phagocytic, is present in young unfiltered cultures; but if present at all, is only found in small quantity in filtrates. The presence of this antigen in an *inoculum* evokes the development of "opsonins" which are specific to the Types.

(c) The *spasm-producing toxin*—an antigen which is filtrable and which, in laboratory animals at least, does not appear to be specific to the Types.

These findings suggest, that improvement might be looked for in serum prophylaxis and serum therapeutics from the employment of sera possessing anti-bacterial as well as antitoxic properties.

The demonstration of specific opsonic activity further suggests, that the typing of the bacilli may be of some import in the pathology of the disease.

III. In Section V the following subjects are considered:

- (i) The mechanism of infection in tetanus.
- (ii) The influence of antitoxic serum on infection with *B. tetani*, as contrasted with its influence upon intoxication with the products of that organism.



(iii) The relative value of *antitoxic* and *anti-bacterial* sera for prophylactic use.

(iv) The demonstration of "*whole animal*" immunity specific to the Types. This is the natural corollary of the experiments undertaken *in vitro* for the demonstration of *anti-bacterial* bodies specific to the Types. From the results obtained the following conclusions may be drawn:

1. That the *spasm-producing* toxin of *B. tetani*, when employed in sub-lethal doses, does not produce sufficient local devitalisation of tissue to permit of the growth of *B. tetani* when inoculated along with it.

2. That the toxin of *B. Welchii*, and to a less extent that of *Vibrio septique*, when used in sub-lethal doses, do produce sufficient devitalisation of tissue to allow of the development of tetanus infection. Antitoxins to the products of these organisms protect animals against infection with *B. tetani* when such products are used as tissue debilitants.

3. The protection afforded by tetanus antitoxin can only be partial, for, if the degree of tissue devitalisation be great, antitoxin used prophylactically fails to prevent the occurrence of tetanus.

4. The nature of the substance used for producing devitalisation of tissue exerts a profound influence upon the development of tetanus spores in the tissues. In guinea-pigs, saponin produces a lesion which always results in the development of spores of *B. tetani* inoculated along with it. The same reagent fails to initiate tetanus infection in the mouse. Trimethylamine, although it may cause the production of a large slough in mice, only infrequently causes the development of spores of *B. tetani* inoculated along with it. Calcium chloride of such concentration that it produces no obvious local lesion will almost invariably cause tetanus spores to develop in these animals.

5. Just as the products of *B. Welchii* and *Vibrio septique* induce tetanus infection, so certain other relationships, the nature of which is at present not determined, appear to depress the infectivity, or toxogenicity, of certain strains of *B. tetani*.

6. The experiments described in this section suggest, but do not prove, that while *monovalent antitoxic* sera exert no specific neutralising influence on the *spasm-producing* toxins of any one Type, they may nevertheless exert a specific *anti-infective* influence. This *anti-infective* influence is *quantitative*, but not *qualitative* in character.

7. The results so far obtained in the investigation of the relative value of *antitoxic* and *anti-bacterial* sera are equivocal. They suggest, however, that this is worthy of more extended investigation. This cannot be done until larger quantities of specific sera are available.

IV. In Section VI are discussed the results obtained in investigating the influence which various dressings exert upon the anaërobic flora of wounds. From this inquiry it is seen, that the dressings, which we were in a position to investigate, exert but little influence upon the anaërobes present in the wounds.

One important fact emerged from the investigation, namely, that excision of the wound area, irrespective of the dressings employed in treatment, exerts a beneficial influence. Anaërobe infection is relatively less when this procedure is employed and convalescence is established at an earlier date than when other methods have been used.

In connection with this work, experiments were undertaken to determine the antiseptic value of certain of the aniline dyes. It is found that the antiseptic activity of these may be greatly reduced in presence of fresh tissue, although their activity may not be reduced in presence of serum.

Tetanus bacilli may be found in wounds at any time during the process of healing. In one instance *B. tetani* was recovered from a wound 882 days after the infliction of the injury. It is sometimes difficult to demonstrate the presence of *B. tetani* in such circumstances, and several swabs may have to be taken before their presence is appreciated by the observer.

V. Examination of the blood of patients suffering from tetanus showed that agglutination could not be employed as an aid to the diagnosis of the disease—Section VII.

VI. In Section VIII is discussed the possible relationship which might exist between the serological Type of a tetanus bacillus, and the haemagglutinating Type of the individual from whom it was isolated. No relationship could be shown to exist between the two.

VII. In Section IX are discussed two interesting cases of abdominal tetanus.

VIII. In Section X are discussed the results obtained in making an examination of human faeces with a view to demonstrating the prevalence of the various Types of *B. tetani* in the human intestine. Unfortunately, owing to the numerous other and more pressing problems under investigation, this work could not be pursued with the vigour necessary to obtain adequate information which would permit of definite conclusions being drawn.

In conclusion we wish to record our thanks to our friends and colleagues who have assisted in the prosecution of this work by their advice and kindly criticism. To Major-General Sir David Bruce, K.C.B., F.R.S., A.M.S., we are especially indebted for inviting us to undertake the investigations, to Lt.-Colonel M. H. Gordon, C.M.G., R.A.M.C., for freely offered advice and encouragement.

That section of the work which deals with experiments on infection was greatly assisted by valuable suggestions offered us by Dr F. Ransom, and Capt. W. E. Bullock, R.A.M.C.

We also owe a debt of gratitude to Lt.-Colonel Sir Alfred Pearce Gould, K.C.V.O., R.A.M.C. (T.), of the 3rd London General Hospital, and to Bt.-Colonel R. J. C. Cottell, R.A.M.C., of King George's Red Cross Hospital, for giving us access to the cases which furnished material for the investigation described in Section VI of the Report; also to Dr Thompson and Capt. Rhodes-Harrison, R.A.M.C. (T.), for their interest and assistance in the

prosecution of the work. In no less degree do we appreciate the assistance of the surgeons and sisters in the various wards of these Institutions.

To the Governing Body and Director of the Lister Institute of Preventive Medicine we are greatly indebted for placing laboratory accommodation at our disposal and for generous financial assistance.

We also beg to thank Professor F. W. Andrewes, F.R.S., who very kindly arranged for the collection of faeces from civilian patients at St Bartholomew's Hospital.

Pte H. C. Wilson, R.A.M.C., by his untiring devotion to the work and by his help in many directions, has really made possible the successful prosecution of the investigation. We desire to record our appreciation of his assistance and warmly to appraise his energy and resource.



THE LABORATORY DIAGNOSIS OF TYPHUS FEVER.  
FURTHER OBSERVATIONS ON THE VALUE AND ON THE  
SIGNIFICANCE OF THE WEIL-FELIX REACTION.

By N. HAMILTON FAIRLEY, O.B.E., M.D., MAJOR, R.A.M.C.  
*Pathologist, 14th Australian General Hospital.*  
*Lecturer and Examiner in Parasitology, Egyptian University.*

CONTENTS.

	PAGE
1. Concerning certain etiological aspects of Typhus: Typhus fever a septicaemia—The virus in the peripheral blood; its filterability and its transmission to animals and man—The Spirochaete of Futuki—The <i>B. typhi-exanthematicus</i> of Plotz— The Weil-Felix reaction . . . . .	204
2. Characteristics of the <i>B. proteus</i> used in this investigation . . .	205
3. Agglutination phenomena in Typhus Fever: In the febrile period of Typhus Fever—In convalescence of Typhus Fever—Time incidence in the appearance of agglutinin— The agglutinin content of the patient's blood, and its bearing on the prognosis—Control reactions with non-typhus sera . . . .	205
4. The complement-fixation reaction: Technique; preparation of reagents and antigen from <i>B. proteus</i> — Investigation of the sera of typhus patients by the complement- fixation reaction—Investigation of non-typhus sera—Investiga- tion of the serological response in monkeys inoculated with living <i>B. proteus</i> (Table I)—Investigation of the serological response in man inoculated with <i>B. proteus</i> (Table II) . . . .	208
5. The significance and explanation of the Weil-Felix reaction: The secondary invasion hypothesis—The hypothesis of secondary non-specific agglutination ( <i>Nebenagglutinin</i> ) . . . . .	213
Conclusions . . . . .	215

FOLLOWING the capture of Jerusalem and in the early months of 1918 Capt. C. M. Craig, R.A.M.C., obtained a culture of a proteus-like bacillus from the civil Jewish bacteriologist of that city. He was informed that this organism was being extensively used by German and Austrian bacteriologists on the Eastern front for the diagnosis of Typhus Fever. After making some preliminary observations on its agglutination by the sera of typhus patients he forwarded the culture on to this laboratory for further investigation, the preliminary account of which was published by Craig and Fairley (21 Sept., 1918, *Lancet*, pp. 385-6).

In the following pages I propose to put on record a *further series* of personal observations fully supporting the conclusions reached in the above-mentioned report.

#### 1. CONCERNING CERTAIN ETIOLOGICAL ASPECTS OF TYPHUS FEVER.

Clinically, Typhus Fever must be regarded as a septicaemia. The typical course of the disease, the temperature chart, the dark macular or petechial rash, the markedly toxic condition of the patient all combine to impress this conception upon even the casual observer. Furthermore, as Nicolle first demonstrated, the virus of Typhus is present in the peripheral blood during the pyrexial period, for it may then be transmitted directly to certain species of monkeys and to guinea-pigs by subcutaneous injection of whole blood. Under natural conditions, however, the virus is transmitted from man to man by the louse (*Pediculus humanus*)<sup>1</sup>. Ricketts and Wilder maintain that blood is not infectious if passed through a Berkefeld filter, but Nicolle reports that the virus is filterable. Many organisms have been suggested as the causative agent in the production of this disease, especially bacillary, diplobacillary, and diplococcal forms. As recently as 1917 Futuki has described a spirochaete resembling *T. pallidum* as occurring in the kidney and supra-renal glands of patients dying of Typhus. The same organism was found in the kidney of a monkey inoculated with Typhus Fever.

The organism having most claim to pathogenicity appears to be the *B. typhi-exanthematici* isolated by Plotz<sup>2</sup> from the blood of typhus patients in 1915. It is a small pleomorphic Gram positive non-motile bacillus growing anaerobically in ascitic fluid sterile tissue media. The sera of convalescents from typhus, according to Rabinowitch, show both positive complement fixation and agglutination reactions for this organism when used as antigen. Olitsky<sup>3</sup>, in his recent immunological studies on this bacillus, has confirmed these serological reactions and has shown the presence in typhus serum of the following specific antibodies, *i.e.* agglutinins, precipitins, immune opsonins, anaphylactic substances and complement-fixing bodies. Numerous other organisms have been described by various investigators until the literature on the etiological aspects of Typhus Fever has become as obscure as it is voluminous. Furthermore despite all past observations on the subject no laboratory test of diagnostic value had been evolved. The recent claims, therefore, of Weil and Felix<sup>4</sup> regarding the diagnostic reliability of their agglutination reaction have become a matter of importance to the clinician and the pathologist alike.

<sup>1</sup> A full account will be found in Nuttall (1917), "The part played by *Pediculus humanus* in the causation of disease." *Parasitology*, vol. x. pp. 44-57, 74-75.

<sup>2</sup> Plotz, Olitsky and Baehr (1915), *Journ. Infect. Dis.* xvii. 1-68.

<sup>3</sup> Olitsky (1917), *Journ. Immunology*, ii. 363.

<sup>4</sup> Weil and Felix (1917), *Wien. klin. Wochenschr.* xxx. 393-9; Viteček, *Ibid.* 967-972.



## THE WEIL-FELIX REACTION.

In 1915 Weil and Felix, while investigating a group of cases thought to be enterica but giving negative Widal reactions, isolated an organism from the urine which was agglutinated by the patient's serum in a dilution of 1 in 200. The serum of nine other cases, all of which proved to be Typhus Fever, likewise agglutinated this organism which was identified as belonging to the proteus group. This organism was named the X 2. Later the same observers isolated from the urine a second strain of a similar bacillus known as the X 19, which differed from X 2 in being agglutinated in a very much higher titre by the sera of typhus patients (1 in 2000). A number of other German and Austrian workers<sup>1</sup> have reported favourably on the reaction during the past year.

## 2. CHARACTERISTICS OF ORGANISMS USED IN THE INVESTIGATION.

The organism utilised in the following investigation proved to be a Gram negative slightly motile short bacillus. It grows anaerobically on all ordinary media such as agar and MacConkey's media, and liquefies gelatin. It produces acid and gas in glucose, saccharose, maltose and mannite, but does not ferment dulcete or lactose. It produces indol freely and acid without clot formation in milk. Subcutaneous inoculation with 2 c.c. of a 24 hours' broth culture proved non-lethal to guinea-pigs.

## 3. AGGLUTINATION PHENOMENA IN TYPHUS FEVER.

Every case in the following series was a typical clinical case of Typhus Fever. In seven cases complications existed at the time the blood was collected for examination. In three cases broncho-pneumonia was present; in another case there was a pelvic cellulitis secondary to perforation of an ulcer of the rectum; in three other cases parotitis had supervened.

The blood picture was investigated in twelve uncomplicated cases. Absence of leucocytosis, or a definite leucopaenia, was the rule during the first week, but this was generally replaced by a moderate grade of leucocytosis during the second week (10,500 to 14,000 per cmm.). The differential count in the second week showed an absolute and relative increase in the polymorphonuclear neutrophile elements and a decrease in the eosinophile cells.

The agglutination was carried out in all cases on a Garrow's agglutino-meter<sup>2</sup>, the results were read after five minutes rocking. Controls were used in every instance. In a number of cases concomitant observations were made by using the macroscopic method of tube agglutination and incubating at 37° C. for four hours.

With the prevailing temperature conditions of Egypt I have always found the Garrow's agglutino-meter a most reliable instrument. Economy of material,

<sup>1</sup> Sterling and Sterling, *Wien. klin. Wochenschr.* xxx. 972-4; Ballner and Finger, *Ibid.* 966-7; Dadez and Kvahelsha (1917), *München. med. Wochenschr.* lxiv. 1379-1381.

<sup>2</sup> Garrow (1917), *Lancet*, i. 262.



the rapidity with which results can be read, and the elimination of the feeble agglutinating power of certain sera upon this organism, are the great advantages of this method.

Using this instrument I regard an agglutination of 1/40 as diagnostic of Typhus Fever. Up to the 6th day of the disease an agglutination in a dilution of 1 in 20 should be regarded as sufficient evidence on which to isolate a case.

Analysis of Cases.

(a) Thirty-five cases were examined during the pyrexial period. The titres of the agglutinations were as follows:

Day	Cases	Titre
5th	1	1/40
6th	2	1/80, 1/320
8th	3	1/80, 1/80, 1/1280
9th	3	1/20, 1/40, 1/640
10th	9	1/80, 1/160, 1/160, 1/320, 1/640, 1/640, 1/1280, 1/1280
11th	2	1/40, 1/1280
12th	5	1/20, 1/80, 1/80, 1/640, 1/1280
13th	5	1/640, 1/640, 1/1280, 1/1280, 1/1280

In five febrile cases the exact day of the illness was not known. The titres of the agglutinations obtained were 1/40, 1/320, 1/320, 1/1280, 1/2560.

(b) The sera of twenty-five convalescent cases gave positive reactions as follows:

Day of convalescence	Cases	Titre
1st	2	1/160, 1/1280
2nd	2	1/20, 1/1280
3rd	2	1/40, 1/160
4th	3	1/160, 1/160, 1/640
5th	2	1/40, 1/160
6th	4	1/640, 1/640, 1/1280, 1/2560
7th	1	1/1280
8th	1	1/640
9th	2	1/160, 1/640
10th	1	1/160
12th	1	1/320
14th	1	1/1280
15th	1	1/640
16th	1	1/80
22nd	1	1/320

Progressive agglutination readings were made in four cases as follows:

Case 1.	3rd day, negative	1/20	2 days after crisis	1/20	positive
	6th	„ „	1/20	„ „	„
	8th	„ „	1/20	11	„ „ „ 1/80 „

This case constituted the mildest clinical type of Typhus Fever of the series.

<i>Case 2.</i>	5th day, negative 1/20	9th day, positive 1/160
	7th „ „ 1/40	11th „ „ 1/1280

This patient died of an overwhelming toxæmia on the 12th day of illness.

<i>Case 3.</i>	6th day, positive 1/320
	8th „ „ 1/640
	10th „ „ 1/640
	12 days after crisis positive 1/160

This patient on the 6th day of illness developed broncho-pneumonia.

<i>Case 4.</i>	6th day, positive 1/80
	8th „ „ 1/80
	13th „ „ 1/640

This patient recovered.

(c) Time incidence in the appearance of agglutinin:

In the series of cases under review the observations made during the first week of the disease are very limited, but in the preliminary report of Capt. C. M. Craig and the writer, the agglutination reactions in twenty-five cases between the 4th and 7th day of the disease were recorded.

These results were as follows:

Day	Cases	Titre
4th	6	1/10, 1/10, 1/40, 1/50, 1/100, 1/1000
5th	10	1/10, 1/10, 1/10, 1/40, 1/50, 1/50, 1/50, 1/50, 1/80, 1/160
6th	6	1/50, 1/50, 1/50, 1/80, 1/100, 1/320
7th	3	1/10, 1/20, 1/320

During the above-mentioned period, though agglutinin was definitely present, its titre was not nearly as high as in the subsequent course of the disease. In the 2nd and 3rd weeks of the disease (*i.e.* in the 2nd week of the febrile period and in the first week of convalescence) the maximum height of the agglutinin curve is attained, as may be ascertained by a perusal of the preceding tables.

A certain proportion of the cases do not develop agglutinin during the course of the fever. In the present series 5 out of 65, or 7·7 % of the cases, failed to agglutinate *B. proteus* in a dilution of 1 in 20 of patient's serum. In four of these cases the agglutinin content was not investigated during convalescence. The remaining case developed agglutinin during the first week of convalescence.

(d) The agglutinin content of the patient's blood and its bearing on the prognosis:

There appears to be no direct relationship between the amount of agglutinin present in the circulating blood and the clinical aspect of the case. In the present series the mildest case had no agglutinin for *B. proteus* until the 2nd day of convalescence, whereas some of the most fatal cases had well marked agglutination established as early as the 5th day of fever. Other cases dying later in the disease (11th to 13th day) only developed agglutinin twenty-four hours before death.

(e) Control reactions:

The sera of 120 known negative cases have been examined for agglutination against this proteus-like organism.

Of the protozoal diseases, twenty-five cases of syphilis, thirty cases of relapsing fever, and thirty cases of malaria (twenty-five cases of sub-tertian infection, four cases of benign tertian, and one case of quartan) were examined with negative results.

The bacterial infections which included cases of influenza, pneumonia, undulant fever, and enterica group yielded negative results with two exceptions.

In these two cases (with Garròw's agglutinometer) agglutinations in a titre of 1 in 10 were obtained but the reaction failed with higher dilutions.

Recently Captain C. M. Craig, R.A.M.C., informed me he has observed an agglutination of 1/1000 with *B. proteus* in a definite case of typhoid fever, but he was unable to exclude a previous Typhus infection.

In a group of seven cases of Typhus which were tested against stock culture of *B. typhosus* and *B. paratyphosus* A and B, during the pyrexial period, six yielded negative results. In the other case which had been inoculated with T.A.B. nine months previously, *B. paratyphosus* A and B yielded a positive agglutination with the patient's serum in a dilution of 1/40, while *B. typhosus* yielded agglutination in a dilution of 1/1280. At autopsy infection with *B. typhosus* was definitely excluded in this case.

The sera of convalescent Typhus patients tested against stock culture of *B. shiga*, *B. flexner*, Y, *M. melitensis*, *cholera vibrio*, and *B. coli* all yielded negative results. Similarly *B. proteus* failed to be agglutinated by standard immune sera of *B. shiga*, *B. flexner*, *B. typhosus*, *B. paratyphosus* A and B, and of the cholera serum prepared by the Lister Institute.

#### 4. COMPLEMENT-FIXATION REACTION.

Technique employed; preparation of reagents and of antigen from *B. proteus*.

As the sera of typhus patients agglutinated, in a high titre, a saline suspension of this proteus-like organism, an investigation was carried out by means of the complement-fixation method (Bordet and Gengou, 1901, *Ann. Inst. Pasteur*, xv.):

Complement-fixation reactions are dependent on the fact that when antigen, inoculated serum (? immune body) and complement are mixed together immune body firmly combines with antigen and complement in such a manner that complement can no longer be found free in the mixture. If such a mixture is allowed to stand at a suitable temperature, *i.e.* 37° C., for one hour or more, and to it is added a suspension of red blood corpuscles sensitized with a suitable quantity of haemolytic serum, no haemolysis will take place since there is no free or available complement. This constitutes a positive reaction and proves the presence of specific immune body in the inactivated serum.



If the complement is not fixed then haemolysis ensues; this constitutes a negative reaction and demonstrates the absence of specific immune body in the serum under investigation.

The antigens used were in fresh saline (0.85 % NaCl and 0.5 % phenol) suspension prepared from a twenty-four hours' growth of this organism on agar slopes.

The technique employed was similar to that used in the ordinary *quantitative* Wassermann reaction. Three, six, nine and sometimes twelve minimum haemolytic doses of complement were used in the test.

In the first stage of the reaction, quantities of antigen, immune serum and complement were mixed together for one hour at 37° C. Subsequently sensitized sheep's corpuscles were added and final readings were made after another hour's incubation at 37° C.

The results were recorded as follows:

1.	P + + + +	fixation of 12 M. H. doses of complement		
2.	P + + +	„	9	„ „
3.	P + +	„	6	„ „
4.	P +	„	3	„ „

*Antigen.* The antigens employed in the present investigation were prepared by two methods:

*Antigen A.* This antigen consisted of a fresh saline (*v. supra*) suspension of a twenty-four hours' growth of *B. proteus* on agar slants (this antigen was the one used in fifty-eight cases of Typhus Fever, and in animal and human inoculation experiments).

*Antigen B.* In this method the (fresh) saline suspensions prepared from growths of *B. proteus* on agar slopes (twenty-four hours old), were heated to 56° C. for one hour and then carefully centrifuged. The supernatant suspension was utilised as antigen. (This was used to investigate nine cases of Typical Typhus.) In standardising the antigen it was found advisable never to use more than one-third the anti-complementary dose.

*Haemolytic serum* was obtained from rabbits by injecting intraperitoneally and intravenously sheep's corpuscles in progressively increasing doses. The serum used in these tests was one of high titre (1/4000). To sensitize the sheep's corpuscles four minimum haemolytic doses of amboceptor or haemolytic serum were used. The M.H.D. of the amboceptor was taken to be that amount of haemolytic serum just sufficient to produce in one hour at 37° C. complete lysis in one volume of a 3 % suspension of sheep's corpuscles with four or five M.H.D.'s of complement.

*Sheep's corpuscles.* Equal quantities of sheep's blood were mixed with 2 % sodium citrate in physiological saline. Requisite amounts of this mixture received three washings with nine times the volume of physiological saline, and were finally made up to the equivalent of a 3 % suspension of sheep's corpuscles in the same solution.

*Sensitization of corpuscles.* After four M.H.D.'s of amboceptor had been added, the suspension of corpuscles was placed in the incubator at 37° C. for thirty minutes, and after sensitization kept in the ice-chest till required.

*Patient's serum.* Blood was usually obtained on the day preceding the test, and kept in the ice-chest till required. The serum was diluted with four times its volume of physiological saline, and heated to 55.5° C. for twenty minutes. Heating in this manner destroys complement and inhibits the anti-complementary properties of certain sera.

*Complement.* The complement used was obtained from well-nourished guinea-pigs, and collected under sterile conditions. The M.H.D. of complement was always obtained by preliminary titration. The reagents were measured out by means of Donald's dropping pipettes.

The arrangement of the systems for the final tests was as follows:

Row No. 1.	Antigen	1 vol.	} + 2 vols. saline (0.85 %)
	Patient's serum	1 „	
	Complement (3 M.H.D.'s)	1 „	
Row No. 2.	Antigen	1 „	} + 1 vol. saline
	Patient's serum	1 „	
	Complement (6 M.H.D.'s)	2 vols.	
Row No. 3.	Antigen	1 vol.	}
	Patient's serum	1 „	
	Complement (9 M.H.D.'s)	3 vols.	
Row No. 4.	Patient's serum	1 vol.	} + 3 vols. saline
	Diluted complement (3 M.H.D.'s)	1 „	

Row No. 4 serves as a serum control and any anti-complementary tendency in each serum examined is thereby demonstrated.

Additional controls used in the test were:

- (1) Antigen control, *i.e.* 1 vol. of antigen and 4 vols. of saline (0.85 %).
- (2) Antigen 1 vol., pooled negative serum 1 vol., 3 vols. of saline (0.85 %).
- (3) Where possible a sure positive serum was included in the series (*i.e.* a monkey inoculated with *B. proteus*).

#### ANALYSIS OF RESULTS.

##### *Complement-fixation reactions in the sera of typhus patients.*

In all fifty-eight sera collected from typhus cases in different stages of the disease were examined. Their agglutination reactions were as follows:

In	1 case	the titre was	1/2560
„	13 cases	„ „ „	1/1280
„	15 „	„ „ „	1/640
„	6 „	„ „ „	1/320
„	9 „	„ „ „	1/160
„	6 „	„ „ „	1/80
„	5 „	„ „ „	1/40
„	3 „	„ „ „	1/20



As far as possible blood was collected from the cases during the second week of fever or in the first week of convalescence (*i.e.* 8th–21st days), during that period when the agglutinin content was at a maximum.

In fifty-five out of fifty-eight of the cases examined the complement-fixation reactions were negative. In one case a P+++ reaction was obtained, in another case there was a similar reading, but the control showed the serum to be anti-complementary. In one other case a P++ reaction was recorded.

*Examination of non-typhus sera.*

Eighty-three sera from non-typhus cases were examined. Of these eighty yielded negative results and three yielded pseudo-positive reactions. One case of rheumatic fever gave a P++++ reaction, and two cases of syphilis yielded a P++ reaction. The sera of twelve other cases of syphilis yielding positive Wassermann reactions were negative.

*Investigation of the amount of complement fixed by pooled typhus serum in the presence of B. proteus as antigen.*

A pooled serum was prepared from nine cases of Typhus all of which yielded high titre agglutinations. The amount of complement fixed by this serum in the presence of *B. proteus* as antigen was found to be identical with the amount fixed by a pooled serum prepared from eleven non-typhus patients. Less than 1½ M.H.D.'s of complement were fixed in each system.

*Conclusion.* It follows from the above experiments that the formation of agglutinin in the blood of typhus cases for this *particular kind of B. proteus* is not accompanied by the formation of immune body, as indicated by the complement-fixation reaction.

EXPERIMENTS ON ANIMALS.

Ten monkeys were experimented on (seven *Cercopithecus* and three *Macacus rhesus*).

Of these ten monkeys four were used as controls throughout the investigation. The six others were injected sub-cutaneously with one dose of from 1 to 2 c.c. of a twenty-four hours' broth culture of *B. proteus*. In only one of these monkeys was a second injection given and this was to a monkey labelled "Z" at an interval of ten days following the first injection.

In every case, prior to inoculation, the serum of the monkey was investigated for complement fixation and agglutination reactions against this organism. All had negative complement fixation, and all yielded negative agglutination reactions in dilution of one in ten with one exception only. In this case (monkey "W") a positive agglutination of monkey's serum in a titre of one in ten was obtained but the reaction vanished in a higher dilution of 1 in 20. None of the inoculated monkeys showed obvious signs of sickness.

A perusal of Table I will show the serological reactions of monkeys inoculated sub-cutaneously with broth cultures of this organism.



An analysis of the protocol will also show that in all cases a positive complement-fixation reaction was obtained. In five out of the six cases this was present within twelve days of inoculation. In the sixth case (monkey "D") the complement-fixation reaction was negative on the ninth day, though it became positive on the eighteenth day. The agglutination response in this case was not as intense as usual.

In all these cases accompanying the appearance of agglutinin in the blood serum of the inoculated monkeys, immune bodies were produced which had the property of fixing complement in the presence of its specific antigen, namely *B. proteus*.

INOCULATION OF MAN WITH *B. proteus*.

In order to observe the serological and clinical effects produced by sub-cutaneous injections of *B. proteus* two of the laboratory staff (R. C. S. and N. H. F.) were inoculated sub-cutaneously with a saline suspension of 3000 million living *B. proteus*. In both cases a systemic reaction followed (generalised aches and pains, headache, fever 100–102° F.); but these symptoms disappeared within forty-eight hours. A local inflammation occurred at the site of inoculation. In one case resolution without suppuration followed, in the other, on the ninth day, the site of inoculation was incised. A small amount of *sterile pus* was present.

Table I.

*Serological Reactions in Infected Monkeys.*

Days after inoculation	MONKEY "Z"		MONKEY "W"		MONKEY "X"		MONKEY "V"		MONKEY "D"		MONKEY "A"	
	Agglutination	Complement fixation	Agglutination	Complement fixation	Agglutination	Complement fixation	Agglutination	Complement fixation	Agglutination	Complement fixation	Agglutination	Complement fixation
4	1/20	—	—	—	—	—	—	—	—	—	—	—
6	1/160	P++++	1/320	—	—	—	—	—	—	—	—	—
9	1/640	—	1/2560	P++++	1/1280	P++++	—	—	1/150	Neg.	—	—
12	1/1280	P++++	—	—	—	—	1/640	P++++	—	—	1/160	P++
18	1/1280	P++++	—	—	1/640	—	—	—	1/320	P++++	—	—
25	—	—	1/320	P++++	1/320	P++++	—	—	1/320	P++++	—	—
35	1/640	P++++	—	—	—	—	—	—	—	—	—	—
53	1/40	Neg.	1/160	P++	1/160	P++	—	—	1/20	Neg.	—	—

Table II.

*Serological Reactions in Man inoculated with living B. proteus.*

Days after inoculation	Case 1		Case 2	
	Agglutination	Complement fixation	Agglutination	Complement fixation
Before	Neg.	Neg.	Neg.	Neg.
6th day	1/360	P+ +	1/160	P+
13th "	1/160	P+	1/640	P+ +
18th "	1/80	Neg.	1/640	P+ +

The result of the serological reactions observed will be found in Table II. In both cases agglutinin and complement fixing bodies appeared in the circulating blood a few days after inoculation.

##### 5. SIGNIFICANCE AND EXPLANATION OF THE WEIL-FELIX REACTION.

Naturally the significance of the Weil-Felix reaction has caused much discussion. The general consensus of opinion as quoted in a leading article in the *British Medical Journal* (12. I. 1918), including that of Weil and Felix themselves, is that *B. proteus* is not the real cause of Typhus, but a specific secondary invader of the body which accompanies the unknown virus of the disease.

That *B. proteus* is not the causative agent in Typhus is indicated by the facts that (1) it cannot be isolated from blood cultures made during the pyrexial period; (2) it is non-pathogenic to man and monkeys when inoculated sub-cutaneously.

*The secondary invasion hypothesis*, while superficially plausible and while accounting for the presence of high titre agglutinins in the blood serum of typhus cases, is incompatible with the following facts:

(1) Systematic cultural investigations of the blood of typhus cases over different periods of the disease and also of the urine, save in very rare cases<sup>1</sup>, yield negative results, even though *B. proteus* grows aerobically and very readily on all the ordinary laboratory media; furthermore it must be remembered that in any case systematic cultural examinations of the urine of any large series of cases will reveal an occasional *B. proteus* infection, hence the cultural findings of Weil and Felix are by no means conclusive.

(2) Serological investigations of typhus sera, while showing the presence of agglutinin for *B. proteus*, have failed to demonstrate the presence of immune body; whereas sub-cutaneous injections into monkeys and man have been followed by the production of agglutinin and complement fixing antibody.

My observations here are entirely in *disagreement* with those of Wagner (1917, *München. med. Wochenschr.* p. 792), who claims to have obtained positive results with the complement-fixation test using *B. proteus* as antigen in five out of six cases of Typhus so investigated.

*Per contra my results showed:*

(1) No increased tendency for fixation of complement by a pooled typhus serum in the presence of *B. proteus* antigen over that quantity fixed by a pooled negative serum under similar conditions.

(2) Fifty-five out of fifty-eight cases of definite Typhus Fever yielded negative complement-fixation reactions.

(3) Using an identical technique, monkeys and man after sub-cutaneous inoculations invariably yielded positive complement-fixation reactions.

<sup>1</sup> Craig and Fairley, *loc. cit.*



In consequence of these findings, and in contra-distinction to the generally accepted view, I hold that the only satisfactory explanation of the Weil-Felix agglutination reaction is to regard the phenomenon as due to a secondary or heterologous agglutinin (*Nebenagglutinin*).

It would be a matter of considerable interest to investigate the residual agglutinin in typhus sera after saturation with suspensions of *B. typhi-exanthematici* Plotz and *B. proteus* Weil and Felix. Additional data might thereby be obtained concerning the etiological significance of both micro-organisms.

Posselt and Sagasser (1903, *Wien. klin. Wochenschr.* No. 24) showed that in immunisation there is not only an increase in the amount of agglutinin for the organism injected but also of secondary agglutinins (*Nebenagglutinine*) which act on other organisms. Thus they found that while the serum of a guinea-pig immunised against *B. typhosus* contained specific agglutinin for that organism, even in a titre of 1/12,000, it also had developed secondary heterologous agglutinins for *V. cholerae* of a titre of 1 in 4500, and for *B. dysenteriae* of a titre of 1 in 4000.

Later Ballner and Sagasser showed that at times these secondary agglutinins are markedly increased and that inoculation with certain organisms like *B. tetani* and Friedländer's bacillus, while leading to the formation of but few specific agglutinins (*Hauptagglutinine*), produced numerous secondary heterologous agglutinins.

A review of the literature certainly leads to the conviction that the sera of typhus cases must be particularly rich in these secondary agglutinins.

Thus agglutinins in typhus sera have been described by the following observers for the following micro-organisms:

(1) For *B. typhi-exanthematici* by Plotz (*loc. cit.*).

(2) For *Bacillus* "U" by Wilson (*Journ. Hygiene*, 1909, ix. 316-337; *ibid.* 1910, x. 155). This organism is a variant strain of *B. coli* and was isolated from the faeces of a case of Typhus during the Belfast epidemic of 1908. Wilson attributes this agglutination to the presence of secondary heterologous agglutinin.

(3) For *Bacillus typhosus*, Wilson (*loc. cit.*) reports positive agglutination in a titre of 1 in 50 as existing in the sera of eighteen out of thirty-one cases of Typhus, and concluded that the Widal reaction was of no value in distinguishing Typhus Fever from Typhoid.

(4) Hornicki, in Manchuria, isolated an organism allied to *Bacillus* "U" of Wilson from the urine and faeces of typhus cases and reported positive agglutination reactions with the sera of typhus patients.

(5) Weil and Felix (*loc. cit.*) have described two strains of *B. proteus* (the X 2 and the X 19) which have already been referred to in detail.

Wherein lies the explanation of this almost promiscuous agglutinating action of typhus serum on a number of biologically distinct species of micro-organisms?



Surely it is impossible for *all* these organisms to be constant secondary invaders in Typhus Fever. Is it not more rational to think that the virus or specific agglutinin of typhus, whilst stimulating homologous receptors or specific agglutinins, also has the property of stimulating other closely related receptors of secondary agglutinins, which agglutinate micro-organisms of different biological strains? Such a hypothesis would afford an explanation not only of the presence of agglutinin for *B. proteus* and the absence of specific immune body for that organism in the sera of typhus cases, but also of the wider agglutinating properties possessed by typhus sera in general.

Furthermore such a conclusion need not detract from the value of the Weil-Felix reaction as a laboratory aid to the diagnosis of Typhus Fever.

The fact that the Wassermann reaction is not a specific test for syphilis, in the strict immunological sense, has not diminished its practical application as a diagnostic test for that disease.

In similar fashion the appearance in the sera of typhus patients of the agglutinin for an organism, which has no apparent relationship to the disease, need not bias the student against the great diagnostic value of this reaction.

#### CONCLUSIONS.

(1) The Weil-Felix agglutination reaction has again proved, in a further series of cases, to be a very reliable laboratory aid to the diagnosis of Typhus Fever.

(2) Frequently the reaction becomes definitely established during the first week of the disease. The maximum agglutination readings are obtained during the second week of fever and during the first week of convalescence (*i.e.* 8th to 21st days).

(3) Of sixty-five cases of definite Typhus Fever sixty-three or 94 % yielded positive agglutination reactions.

(4) Of 120 non-typhus sera no case yielded positive agglutination in a dilution of 1 in 20, utilising Garrow's method of agglutination. In two cases an agglutination in a dilution of 1 in 10 was obtained. On the Garrow's agglutinator a positive agglutination of patient's serum in a titre of 1 in 40 may be regarded as diagnostic of Typhus Fever. A positive reaction in a dilution of 1 in 20 of patient's serum may be regarded as sufficient evidence on which to isolate a case during the first week of illness.

(5) The appearance of the agglutinin in the sera of typhus cases for the *B. proteus* utilised in the present investigation is not accompanied by the formation of specific immune body. On the other hand living cultures inoculated sub-cutaneously into monkeys and man are followed, not only by the appearance of agglutinin, but also by the production of immune body as revealed by the complement-fixation test, utilising *B. proteus* as antigen.

(6) The hypothesis that *B. proteus* is a constant secondary invader accompanying the unknown virus of Typhus Fever lacks confirmation and is

incompatible with certain ascertained facts. The Weil-Felix reaction is dependent on the presence in typhus sera of a secondary non-specific agglutinin which has the property of agglutinating this *proteus*-like organism.

#### ACKNOWLEDGMENTS.

My best thanks are due to the Directors of the Infectious Fevers' Hospitals at Cairo and Port Said, Drs Samy Sabonghy and Haywood, respectively, for placing their clinical material at my disposal.

I am indebted to Captain C. M. Craig, R.A.M.C., Major P. H. Bahr, D.S.O., R.A.M.C., and Lieut-Colonel C. B. Blackburn, A.A.M.C., for assistance during this investigation and to Sgt R. C. Stephens, who volunteered to be inoculated with living cultures of *B. proteus*.

For permission to publish this additional report I am indebted to my C.O., Colonel Walter Summons, A.A.M.C.

FURTHER EXPERIMENTS IN THE ETIOLOGY OF  
DENGUE FEVER.

BY

J. BURTON CLELAND, M.D., CH.M. (SYD.), Principal Microbiologist, Micro-  
biological Laboratory, Department of Public Health, Sydney,

AND

BURTON BRADLEY, M.B., CH.M. (SYD.), D.P.H. (LOND.), Director Labora-  
tory of Microbiology and Pathology, Brisbane; late Assistant Micro-  
biologist, Department of Public Health, Sydney, and Hon. Pathologist,  
Royal North Shore Hospital of Sydney.

(Clinical notes by W. MACDONALD, M.B., CH.M., Medical Superintendent,  
Liverpool State Hospital.)

(With 9 charts.)

(From the Laboratories of the Department of Public Health and of the Royal  
North Shore Hospital of Sydney.)

CONTENTS.

	PAGE
Introduction . . . . .	217
I. Injection experiments . . . . .	219
II. Mosquito experiments . . . . .	232
III. Animal experiments . . . . .	233
Appendix. Detailed report of the cases dealt with in the series of injection experiments . . . . .	234
References . . . . .	254

INTRODUCTION.

In a previous communication(1), after reviewing the literature of the subject, and especially the claims as to the transmission of dengue by *Culex fatigans*, we were able to show conclusively that in Australia the Yellow Fever mosquito, *Stegomyia fasciata*, is capable of transmitting the virus of dengue. Whether or not other mosquitoes can also play a part we cannot say, although epidemio-  
logically we think this unlikely. We also showed that the incubation period was certainly over five days, and usually between five and a half and nine and a half days, in our mosquito cases. We showed also that the virus could be transmitted by the subcutaneous inoculation of blood.



In a further communication (2) we showed *inter alia*:

1. That the virus of dengue may be present in the whole blood, the serum, the washed corpuscles or the citrated plasma.
2. That the virus was present in the blood on the second and third days of the disease (24 to 48 hours after onset), and possibly as late as the eighth day (about 168 hours after onset). Some evidence was adduced to show that the virus was not present in the blood on the fourteenth day of the disease.
3. That our filtration experiments were not conclusive. There was one apparently successful case out of five experiments. In some of these, for other reasons, the result might have been expected to be negative.
4. That the incubation period varied from six to nine days.
5. That immunity to inoculation was present twenty-four days after recovery from dengue.
6. That blood containing the virus may retain its infectivity in a cool place outside the body for at least seven days.
7. That the infection of dengue could be conveyed to at least four "generations" of artificial (injected) cases.

The present series of experiments is the outcome of a project which to a great extent has not materialised. We had intended to establish a chain of artificial dengue cases in volunteers, to keep the virus alive in the series by successive injections, and with this virus to perform as it were a series of side-chain experiments on other volunteers to obtain certain special aspects of the problem. We had made arrangements to breed out mosquitoes and had devised experiments to confirm our previous findings and to determine several important points as to the nature of the transportation of the virus by these insects. We had also planned to conduct experiments as to the nature of the virus in man, and especially as regards its filtrability, its resistance to outside influences, its persistence in the blood and its distribution in the elements of the blood.

Certain facts combined caused us to greatly curtail our scheme and eventually the virus-chain ran out and further experiments were abandoned. This was chiefly due to the unexpected difficulty of obtaining volunteers, even with a considerable monetary inducement. The mosquitoes which we were able to breed for some reason did not bite well and died off rapidly.

This should be borne in mind in considering the experiments herein described. At first sight, in several instances, we seem to have neglected opportunities for experiments, whereas in reality we had no volunteer available at the time.

Again, in two experiments, the finding of a positive complement fixation reaction for syphilis in the volunteer prevented further utilisation of the virus in his blood.

In spite of these difficulties we have been able to obtain further information on certain points as to the dengue virus.

The present experiments show in brief:

1. That it is possible by subcutaneous injection of blood, or derivatives of blood, from a dengue patient to transmit the disease to a healthy non-immune, and from this case in

a similar way to derive a series of experimental cases. In the present communication the original virus obtained from a natural case has been passed successively through four "generations" of artificial cases, with a doubtful positive case in the fifth "generation."

2. That the virus under certain conditions may be passed through a Pasteur-Chamberland F. filter which at the same time is keeping back ordinary organisms (staphylococci, colon bacilli).

3. That the virus does not appear to be specially contained in any one element of the blood. Carefully washed corpuscles, citrated plasma, and serum free from corpuscles all contain the virus. It is not absolutely certain, however, in the case of serum that the virus may not obtain access to this by the breaking up of corpuscles.

4. That the virus is resistant to conditions outside the body for several days (99 hours).

5. That in the blood of the dengue patient the virus was present in one case 18 hours after the onset, in one case 67 hours and in another as late as 90 hours, whilst several cases show it to have been present at 57 hours and less after the onset. It was not found in a case 130 hours after the onset.

6. That the incubation period, while variable and not shorter than  $4\frac{1}{2}$  days, is usually from 6 to 8 days, but may be as long as 15 days.

7. That immunity may be present to injection 229 days after the onset of a previous attack of dengue.

8. That, in one experiment made, no evidence could be found that *Culex fatigans* can transmit the virus of dengue.

9. That, in one experiment made, no evidence was found that the blood of a guinea-pig contained the virus seven days after its inoculation.

10. That no result followed inoculation of guinea-pigs or rabbits, and no pathological lesions were found in inoculated guinea-pigs and no spirochaetes were found in their organs by Levaditi's method.

#### PART I. INJECTION EXPERIMENTS.

Reviewing the cases of the present series, certain peculiarities are shown which may be briefly summarised.

The type of the disease, as in fact is the case in the natural illness, varied very greatly. Certain of our cases were typical of dengue, whilst others were only recognisable as such by the temperature and pulse variation and the nature of the surrounding circumstances. A striking phenomenon is the mildness of the symptoms in the aged volunteers from the Asylum, in contrast to the general severity of the cases in the younger and more active volunteers from our staff and from that of the Royal North Shore Hospital. As will be mentioned later, mild symptoms were often associated with a very definite pyrexia.

Cases 2, 4, 15 and 17 were all classifiable, as regards symptoms, as of "typical rather severe" type. That is to say, they all had marked general body and eye pains and headache with considerable prostration, nausea and malaise, and all looked ill. The rashes in these cases were a prominent feature, and complete recovery was slow, probably in all. Now it is extremely interesting to note that whereas Cases 2 and 4 are at the beginning of our series, Cases 15 and 17 are both fourth generation cases and are separated from the two severe early cases by comparatively mild cases, Case 15 being derived



from Case 10, and Case 17 from Case 11—both Case 10 and 11 being descendants of Case 4. In Case 10, for instance, the volunteer only complained of symptoms for one day and the rash was extremely faint, whereas the descendant Case 15 was feeling very sick for three days, and had a most distinct rash and typical symptoms.

Case 17 was of considerable severity with a distinctly typical rash, whereas Case 11, although showing definite though slight symptoms and a definite slight rash, was altogether much milder than Case 17.

The febrile reaction in most cases was distinct and constituted in some the chief evidence as to the presence of the infection. Some of the cases were not charted in detail but in most cases we have a detailed record of the temperature and pulse from the date when the virus was administered to some time after the end of the febrile phase. The course adopted in this series was to take the temperature twice daily, usually for a week, and if no symptoms or pyrexia declared itself in this period to take the chart four-hourly for the remaining period. The total period of observation was usually between three and four weeks.

A review of Table I and of the charts will indicate, better than a description, the type, duration, and severity of the febrile reaction. It will be seen that the febrile period ranges from somewhat over three days to seven days. In most cases it is easy enough to determine the point of onset of the pyrexia, as also its termination, but occasionally there may be a slight elevation of temperature to about 99° F. for a day or more before or after the termination of the definite fever.

*The degree of fever:* A review of the maximal points in nine of the positive charted cases shows the lowest temperature to be 100·8° F. and the highest 104·6° F. 102° F. would be about the average maximum temperature and in four cases it lies between 102–103° F. The fever seems hardly at all correlated with the symptoms—in some of our cases, especially in Case 14, the want of correlation is especially distinct. This particular case had virtually no symptoms with a temperature of nearly 105° F., which remained high for nearly two days, then fell and rose again to nearly the same level with another short interruption, and then fell abruptly.

*Type of fever:* Although several of the charts are definitely saddle-back in type, and others show a more irregular diphasic variation, others again show an irregular temperature curve. We do not think the type of temperature is at all diagnostic of Australian dengue, either in natural or artificial cases. Although certain cases may show typical saddle-back charts, and as may be seen in our previous papers there may be two separate pyrexial phases, yet other typical cases may show a simple monophasic variation or irregular or plateau types of chart.

*The pulse and its relationship to the pyrexia:* As shown in our previous papers and noted by other observers in natural cases, the pulse of dengue has a distinct tendency to be slow relatively to the degree of fever. This may



Table I.

Case No.	Initials	Result	No. of days during which temp. taken	Incub. period in days	Pyrexia			Days charted after Pyrexia	Pulse during Pyrexia.		Remarks	
					Type	Highest temp. °F.	Duration in days		Bradycardia	Abs.		
												Rel.
2	W. G. A., m.	Pos.	15	8	Irreg.	102·8°	6	1	+	...	++	
3	B. B., m., 34	Neg.	Not taken	...	...	...	...	...	...	...	...	Immune
4	H. G., m., 40	Pos.	Not taken	6½	...	About 102°	...	...	...	...	++	
5	P. N., m., ?	Neg.	33	...	(See history)	...	...	...	...	...	...	Doubtful case.
6	H. D., m., 45	Pos.	28	15	Irreg. diphasic	100·8°	7	6	+	...	++	
7	W. J. I., m., 72	Neg.	21	...	...	...	...	...	...	...	...	
8	J. W. M., m., 63	Pos.	23	10	Irreg. diphasic	101°	7½	6	+	-	+	s
9	J. W., m., 65	Neg.	22	...	...	...	...	...	...	...	...	
10	P. M., m., 45	Pos.	23	9	Irreg. diphasic	102·6°	7	7	++	-	+	s
11	J. D., m., 74	Pos.	21	7	Plateau	102·4°	3 +	11	++	-	+	Later oscillations to 99°
12	P. B., m., 61	Neg.	23	...	...	...	...	...	...	...	...	
13	R. C., m., 58	Pos.	20	8?	Irreg.	100·8°	6?	7	++	+	-	Temp. 99° one day before onset
14	H. W., m., 70	Pos.	17	5	Typical saddleback	104·6°	7	5	++	-	+	s
15	W. M., m., 30	Pos.	12	7	Incomplete chart, prob. irregular	102°	4	1	-	-	++	Whole chart not shown
16	A. A., m., ?	Neg.	21	7	...	...	...	...	...	...	...	
17	N. K., f., 28	Pos.	...	?	No chart	100·3°	...	...	...	...	...	
18	J. F. H., m., 70	Pos.	19	4½	Irreg.	100·8°	5	9	++	...	...	
19	T. B., m., 55	Neg.	23	...	...	...	...	...	...	...	...	
20	C. L., m., 70	Pos.?	...	...	...	...	...	...	...	...	...	
21	H. McD., m. 42	Neg.	Not charted	...	...	...	...	...	...	...	...	

be accompanied by absolute bradycardia (pulses between 50 and 55 are fairly frequent, especially after the pyrexia). Although several of our charts in this series show periods when the pulse is between 50 and 55, definite absolute bradycardia is not a very prominent feature. On the other hand the

relative bradycardia is present in every case of which we have complete charts, and in some of these is very distinct.

*Rashes:* No attempt was made in this series to separate primary and late rashes and detailed descriptions of the rashes are not given. Where it occurred it corresponded to our previous description and was typical of dengue and not readily to be confused with that of any other complaint. In the Asylum cases the rashes were slight, but in the cases among the younger more active volunteers from this staff and elsewhere the rashes were very prominent and characteristic.

Out of eleven cases which were regarded as positive, five showed distinct and typical rashes; one showed a definite, but less distinct, rash, whilst two cases showed only a slight rash which was not prominent enough to be noticed unless specially sought for. The other cases, although they showed an erythematous flushed skin, could not be said to have a rash at any period of their illnesses.

*Symptoms:* These need not be detailed here. Reference to the Appendix will show that they were prominent and typical in the outside cases but mild, sometimes practically absent, in the institution cases. The experience referred to above, in which mild cases intervened in the series between severe cases, teaches us to attach less importance to symptoms in our class of institution volunteers and to rely more on the temperature-pulse variation for determining the positivity of the cases.

#### INFORMATION BEARING ON THE NATURE OF THE VIRUS, ETC., TO BE OBTAINED FROM THE CONSIDERATION OF THE CASES IN THIS SERIES.

Table II indicates the relationship of the human inoculation experiments in this series; it records briefly the nature of the injection, date of injection, date of onset, and the incubation period.

Certain special aspects of the experiments are discussed here (see Appendix, where full experimental and clinical data are recorded).

*Transmissibility of dengue:* As we and others have shown that dengue fever can be successfully transmitted by the blood of a person with the fever to a non-immune and on through several generations, this matter need not be elaborated here. In the present series the virus has been transported through four "generations" of artificial cases. The type and severity of these cases have been discussed above.

*Time during which the blood contains the virus:* Table II shows the duration of the illness in the previous case at the time the material was taken, the period spent by the virus outside the body, and the nature of the material used for injection, correlated with the results of the experiments. This table shows that the virus was present in the blood of the dengue patient as late as 90 hours after the onset in one case; 67 hours in one case; 57 hours in three cases; 47 hours in two cases; 46 hours in one case; 22 hours in two cases; and 18 hours in one case.



Therefore these cases demonstrate that the virus is present in the blood as early as 18 hours after the onset of symptoms and may be present as late as 90 hours after. As we have only one case showing this we cannot be certain that this long period may not be exceptional. However, between 60 and 70 hours after the onset the virus is probably usually present in the blood.

As regards injections followed by negative results, only two of these can be fairly used as indicating an absence of infectivity of the blood. The remaining cases might for other reasons have given a negative result. Case 21, which was negative, injected with the blood of an atypical case taken only a few hours after the onset, will be specially discussed. Cases 12 and 16 were injected with untreated blood. The virus used for the injection of Case 12 was outside the body less than 24 hours and that of Case 16 only a few hours. In Case 12 the blood was taken from Case 4 on the sixth day of the illness (approximately 130 hours after the onset). In Case 16 the blood was derived from Case 10 on the ninth day of the disease (approximately 190 hours after the onset). Both Case 4 and Case 10 were shown to have had the virus in the blood at an earlier stage. From these two cases it may be deduced that the virus was not present in the blood at 130 hours and 190 hours after the onset.

*Summary: The present figures show that the virus may be present in the blood as early as 18 hours and as late as 90 hours after the onset. It was not found in the blood 130 hours and 190 hours after the onset.*

*Resistance of dengue virus outside the body.* The virus seems to resist well for a short period the conditions of a sojourn outside the body. As far as possible when preserving blood for injection we have made use of an ice-chest but in several cases the material was transferred between the laboratories and the hospital with no special precautions, and filtration was performed at room temperature without destroying the virus.

In one instance the untreated blood was preserved, chiefly in the ice chest, for 99 hours before injection and gave rise to a typical case (Case 8). There were several instances where the virus was outside the body about 48 hours, and in one case after 72 hours the virus was possibly still active (doubtful Case 20).

*Summary: The virus outside the body has been found alive, if kept reasonably cool, in several instances up to 48 hours, and in one instance after 99 hours.*

*It is not possible from this series to draw deductions from the negative cases on this question.*

*Filterability of the virus.* In our last communication we reported one apparently positive case and several negative cases after filtration. The single positive case was not fully controlled. In the present series we obtained four negative and three positive results (see Table II).

As regards the negative results, Case 19 can be excluded as the blood was taken, from Case 11, 115 hours after the onset, and our experiments suggest that after this period the virus may have disappeared from the blood.



Table II. *Showing sequence of experiments.*

(1) Dr F. G. (natural case). Ill, 11-1-17 (evening). Blood taken, 13-1-17 (4 p.m.)	
(2) Dr W. G. A. Inj. 0.5 c.c. dil. blood, 15-1-17 (4 p.m.). Ill, 23-1-17, 5 p.m. Incubation period, 8 days. Result— <b>Positive</b> .	(3) Dr B. B., m., 34 yrs. Inj. 0.5 c.c. dil. blood, 15-1-17 (4 p.m.). Result— <i>Negative</i> .
	Blood taken, 24-1-17 (11 a.m.)
(4) H. G., m., 40 yrs. Inj. 1 c.c. diluted blood, 24-1-17 (2.30 p.m.). Ill, 31-1-17, 3 a.m. Incubation period, 6½ days. Result— <b>Positive</b> .	(5) P. N., m. Inj. 9 c.c. diluted filtered blood, 25-1-17 (? 4.30 p.m.). Result— <i>Negative</i> .
	Blood taken, 27-1-17 (11 a.m.)
(6) H. D., m., 45 yrs. Inj. 0.5 c.c. blood, 27-1-17 (5.30 p.m.) Ill, 11-2-17. Incubation period, 15 days. Result— <b>Positive</b> .	
(7) W. J. I., m., 72 yrs. Inj. 2 c.c. diluted filtered blood, 1-2-17 (4.30 p.m.). Result— <i>Negative</i> .	Blood taken, 31-1-17 (about 11 a.m.).
	Blood taken, 2-2-17 (noon).
(12) P. B., m., 61 yrs. Injected 0.3 c.c., 6-2-17 (10 a.m.). Result— <i>Negative</i> .	
Blood taken, 5-2-17 (noon?).	

- (8) J. W. M., m., 63 yrs.  
Inj. 0.8 c.c. blood,  
6-2-17 (3 p.m.).  
Ill, 16-2-17 (2 p.m.).  
Incubation period, 10 days.  
Result—**Positive**.
- (9) J. W., m., 65 yrs.  
Inj. 2.8 c.c. blood filtrate,  
3-2-17 (4.30 p.m.).  
Result—*Negative*.
- (10) P. M., m., 45 yrs.  
Inj. 1.3 c.c. washed corpuscles,  
3-2-17 (4.30 p.m.).  
Ill, 12-2-17 (5 p.m.).  
Incubation period, 9 days.  
Result—**Positive**.
- (11) J. D., m., 74 yrs.  
Inj. 2 c.c. washings,  
3-2-17 (4.30 p.m.).  
Ill, 10-2-17 (4.30 p.m.).  
Incubation period, 7 days.  
Result—**Positive**.
- Blood taken, 13-2-17 (3 p.m.).
- (13) R. C., m., 58 yrs.  
Inj. 2.3 c.c. plasma filtrate,  
15-2-17 (4 p.m.).  
Ill, 23-2-17 (4 p.m.).  
Incubation period, 8 days.  
Result—**Positive**.
- (14) H. W., m., 70 yrs.  
Inj. 0.3 c.c. blood,  
15-2-17 (4 p.m.).  
Ill, 20-2-17 (3 p.m.).  
Incubation period, 5 days.  
Result—**Positive**.
- (15) W. W., m., 25 yrs.  
Inj. 2 c.c. serum filtrate,  
16-2-17 (10 a.m.).  
Ill, 23-2-17 (on rising).  
Incubation period, 7 days.  
Result—**Positive**.
- (16) A. A., m.  
Inj. 1 c.c. blood,  
20-2-17 (5 p.m.).  
Result—*Negative*.
- Blood taken, 20-2-17  
(time not noted).
- (20) C. L., m., 70 yrs.  
Inj. 1.2 c.c. blood,  
27-2-17 (7 p.m.).  
Incubation period (?).  
Result—Doubtful.
- Blood taken, 14-3-17 (time not noted).
- (21) H. McD., m., 42 yrs.  
Inj. 2 c.c. blood,  
16-3-17 (4.30 p.m.).  
Result—*Negative*.
- (17) N. K., f., 28 yrs.  
Inj. 2 c.c. (?) blood filtrate,  
14-2-17 (time not noted).  
Ill between 21 and 26-2-17.  
Incubation period, doubtful.  
Result—**Positive**.
- (18) J. F. H., m., 70 yrs.  
Inj. 2.6 c.c. washings,  
14-2-17 (3 p.m.).  
Ill between 18 and 19-2-17.  
Incubation period, about  
4½ days.  
Result—**Positive**.
- (19) T. B., m., 55 yrs.  
Inj. 2.3 c.c.  
serum filtrate,  
18-2-17 (10 a.m.).  
Result—*Negative*.
- Blood taken, 12-2-17 (3 p.m.).
- Blood taken, 15-2-17  
(about noon).

Table III.

*Showing the data concerning materials used for injection in relation to the results obtained.*

Case	Result	Incubation period in days	Materials injected			Rash	Remarks
			Nature and source	Hours after onset*	Hours outside body†		
(1) Dr F. G., m.	Pos.	Over 5	Naturally acquired			++	
(2) Dr W. G. A., m.	Pos.	8	0.5 c.c. (1 to 2) diluted blood from Case 1	46	48	++	
(3) Dr B. B., m., 34 yrs.	Neg.	...	Same as No. 2	46	48	-	Immune
(4) H. G., m., 40 yrs.	Pos.	6½	1 c.c. diluted (1 to 1) blood from Case 2	18	3½	++	
(5) P. N., m.?	Neg.	...	9 c.c. diluted (1 to 9) blood, filtered, from Case 2	18	About 29	-	
(6) H. D., m., 45 yrs.	Pos.	15	0.5 c.c. undiluted blood from Case 2	90	6½	++	Congenital syphilis
(7) W. J. I., m., 72 yrs.	Neg.	...	2 c.c. diluted (1 to 7) blood, filtered, from Case 4	8	30	-	
(8) J. W. M., m., 63 yrs.	Pos.	10	0.8 c.c. undiluted blood from Case 4	57	99	+	?
(9) J. W., m., 65 yrs.	Neg.	...	2.8 c.c. diluted (1 to 3) blood, filtered, from Case 4	57	28	-	
(10) P. M., m., 45 yrs.	Pos.	9	1.3 c.c. washed corpuscles from Case 4	57	28	+	?



(11)	J. D., m., 74 yrs.	Pos.	7	2 c.c. diluted (1 to 5) citrated plasma from Case 4	57	28	+
(12)	P. B., m., 61 yrs.	Neg.	...	0.3 c.c. undiluted blood from Case 4	130	<24 hours	-
(13)	R. C., m., 58 yrs.	Pos.	8?	2.3 c.c. diluted (1 to 4) citrated plasma, filtered from Case 10	22	49	-
(14)	H. W., m., 70 yrs.	Pos.	5	0.3 c.c. undiluted blood from Case 10	22	49	-
(15)	W. W., m., 30 yrs.	Pos.	7	2 c.c. undiluted serum, filtered, from Case 10	67	22	++
(16)	A. A., m.	Neg.	...	1 c.c. undiluted blood from Case 10	About 190	A few hours	-
(17)	N. K., f., 28 yrs.	Pos.	?	? 2 c.c. diluted (1 to 5) blood, filtered, from Case 11	47	About 48	++
(18)	J. F. H., m., 70 yrs.	Pos.	4½	2.6 c.c. diluted (1 to 2½) citrated plasma from Case 11	47	48	-
(19)	T. B., m., 55 yrs.	Neg.	...	2.3 c.c. diluted (1 to 1) serum, filtered, from Case 11	115	70	-
(20)	C. L., m., 70 yrs.	?	?	1.2 c.c. undiluted blood from Case 15	24	About 72	-
(21)	H. McD., m., 42 yrs.	Neg.	...	2 c.c. undiluted blood from Case 20	?24	48	-
(22)	P. W. P., m., 71 yrs.	Neg.	...	2 c.c. undiluted blood from guinea-pig 3647	See animal experiments		-

\* Hours after onset.—This refers to period elapsing between onset of illness in previous (donor) case and time at which blood was taken from this patient.

† Outside body.—This refers to period elapsing between the time the blood was drawn from donor case and the time when it was injected into the recipient case.

In Case 5 there was some doubt as to whether the result was really negative but there was not a characteristic enough reaction to say that the case was positive. The blood at the time it was taken for filtration was shown to contain the virus (see Case 4). The virus was only outside the body 29 hours.

In Case 7 there was no control to show that the virus was present at the time in the untreated blood from Case 4, but three cases injected with other material taken two days later from the same case (whose blood was used for the filtrate) were positive. It is probable that at the earlier period (8 hours after onset) the blood of Case 4 did contain the virus. The failure in Case 7 cannot therefore be reasonably explained by supposing an absence of the virus at that time in Case 4. The blood was only outside the body 30 hours, and this therefore again cannot be held to account for the failure. The subject will be further considered later.

In Case 9, three cases serve as controls to show that the virus was present in the blood when it was withdrawn from Case 4, and in two of these the virus was outside the body as long as it was in the material used for Case 9 (28 hours) while in the third case the virus was outside the body for a much longer period (99 hours) and was still successfully conveyed.

In the three positive filtration cases—13, 15 and 17—material was taken 22, 67 and 47 hours respectively after the onset of the “mother” cases and was outside the body 49, 22, and about 48 hours respectively.

Before considering the causes determining the failure or success of individual cases we may dwell on the technique of filtration.

### *Technique of filtration.*

The filter used was a small candle about six inches long with a wall one-eighth to one-sixteenth of an inch thick at the top, the inside diameter at the top being ea. one-third of an inch. The filter tapered slightly from top to bottom. It was marked “Chamberland Sme. Pasteur B.E.S.G.D.C.H.B. & Cie, Choisy-le-Roi BES S.G.D.B.,” and stamped “Contrôle,” and on the bottom “F.” It is what is known as the Pasteur-Chamberland F. Filter, which is said to be a coarser grade than the B. type.

For filtration, previously unused candles were fixed in bored rubber corks into the neck of flasks which had side tubes. The whole apparatus was sterilised by steam and cooled, and then attached by the side tube, which was plugged with cotton wool, to the rubber pipe leading to a water pump. The cork was covered with melted paraffin to be sure no leak occurred. The material to be filtered was run into the candle and the pump turned on. When sufficient material had been obtained the plug was removed from the side tube, the end of this flamed and cooled, or wiped with alcohol, and the flask tilted and the filtered material run out into a sterile tube. Generally speaking the pressure at the Health Department Laboratory is not good and the later filtrations were done at the Royal North Shore Hospital Laboratory.

To show that the filters used did not admit the passage of ordinary bacteria, the practice was adopted of mixing organisms with the material to be filtered and testing for their presence by culture in the filtrate.

The organisms added and the nature of the cultural tests were as follow:

*Case 5*, injected with filtered blood from *Case 2* with a doubtful result.

*Organism added.* Emulsion of *B. coli communis* L.I.P.M.

- Cultures.*
1. Broth culture, 1 c.c. of filtrate to 20 c.c. broth. Agar subcultures made from this.
  2. Broth cultures, one loop and two loops.
  3. Agar cultures, one loop and two loops.

*Case 7*, injected with filtered blood from *Case 4* with a negative result.

*Organism used.* Colon bacillus (*B. acidi lactici* type).

- Cultures.*
1. Broth cultures, one and two loops and subcultures from these.
  2. Agar cultures, one and two loops.

*Case 9*, injected with filtered blood from *Case 4*, with a negative result.

*Organism used.* *B. coli communis* L.I.P.M.

*Cultures.* Broth and agar.

*Case 13*, injected with filtered plasma from *Case 10*, with a positive result.

*Organism used.* *B. coli* (type not noted).

- Cultures.*
1. Two loops into 10 c.c. broth.
  2. One loop on agar.

*Case 15*, injected with filtered serum from *Case 10*, with a positive result.

*Organism used.* *Staphylococcus aureus*.

- Cultures.*
1. Two loops into 10 c.c. broth.
  2. One loop on agar.

*Case 17*, injected with filtered blood from *Case 11*, with a positive result.

*Organism used.* Colon bacilli (type not noted).

*Cultures.* Two loops into 10 c.c. broth.

*Case 19*, injected with filtered serum from *Case 11*, with a negative result.

*Organism used.* *Staphylococcus aureus*.

- Cultures.*
1. Two loops into broth.
  2. One loop on agar.

All the cultures in this series remained sterile. The number of organisms added to the material before filtration was large but not specially measured. When an emulsion was used it was made densely milky. In other cases a large loopful of thick scrapings from the cultures was used. For full details of this work see under "Experimental Material," following the record of the case from which the material was derived.

Although in future experiments we would be inclined to use larger amounts of the filtrate than two loops for making the cultures, yet we think in view of the number of organisms added that the above tests show fairly clearly that our filters were restraining the passage of ordinary bacteria under the conditions of the experiments. We think the method of adding the organism to the material to be filtered is a much better control than testing the filter before or afterwards, as, with a variable water pressure, it may not be possible to parallel the conditions of the actual filtration. Moreover the filter itself may be altered in some way by washing and sterilising if controlled before the main filtration, and may be blocked by débris from the filtration if the bacterial test is left until afterwards. No serious effects followed the injection



of any of the filtrates, though a slight rise of temperature and some redness of the arm (possibly due to toxins from the bacteria) were noted next day in some cases.

#### FINAL CONSIDERATION OF THE RESULTS OF THE FILTRATION EXPERIMENTS.

Our results indicate that the dengue virus under certain conditions passes through the Pasteur-Chamberland F. Filter, which at the same time is preventing the passage of ordinary bacteria. The question now arises as to what is the cause of the failure in certain experiments.

Confining ourselves to the present series of tests, Case 19 may be rejected as a test as to the filtrability of the virus for reasons given above. In the negative cases, 5, 7 and 9, however, the virus was almost certainly present in the blood on withdrawal and in Cases 5 and 9 was actually shown to be present in the unfiltered blood from which the filtrate was made.

The sojourn outside the body less than 30 hours in any of the cases, is less than in two of our positive filtration cases and in several of our non-filtration positive cases, so that it is at first sight hard to find any ground for the failure of these cases. It is most unlikely that they were all immunes.

The three unsuccessful cases were obtained with filtered blood which before filtration contained a certain amount of solid material and the filtration was done in the Department's laboratory, and although we have no precise notes on the matter, the filtration was certainly slower than in the later cases, including three successful filtrations. Of the later cases, although in Case 13 filtration was also performed at the Department's laboratory, the filtration took place rapidly, the fluid being free from cellular material. In Cases 15, 17 and 19 filtration took place under better pressure conditions at the North Shore Hospital and filtration was more rapid.

We therefore conclude that the failure of certain of our cases was due to slow filtration and the plugging of the filter pores by solid material through which the fluid had to pass, and the successful cases were due to the more rapid filtration brought about by a higher water pressure and an absence of solid material.

#### *Distribution of the virus in the blood.*

Graham (3), whose results have not received confirmation, described endo-corpuseular bodies in dengue fever. These we regard as artefacts. The fact that the virus has been shown to be filtrable, although it shows that at some stage the virus is very small, does not of course exclude the possibility of an endo-corpuseular phase of a microscopically visible size. Although we have examined blood from dengue cases carefully a number of times with unstained preparations, and with the ultramicroscope, we have not found any evidence of a visible virus. Apart from the question, however, of a visible virus it would be useful if possible to show whether the causative agent was confined to any one element of the blood. It is well to remember, however, that the

distribution may not be the same at all stages. The organism may at one stage, for instance, be endo-corpuscular or endo-leucocytic, and at another stage free in the serum. On the other hand the virus may be a special inhabitant of one or other element of the blood.

Our previous results led us to think that, whatever be the nature of the virus, it was apparently not strictly endo-corpuscular, though we could not exclude the possibility of an endo-corpuscular or endo-leucocytic phase followed or accompanied by a phase in which it was free in the plasma. Our present series of experiments, though not yet completely demonstrative, tend to support our previous view.

It should be remembered, however, that processes designed to effect a separation of the blood elements may at the same time cause some breaking up of the corpuscles and a consequent liberation of a virus. A consideration of the whole circumstances, however, leads us to think that this theoretical breaking up cannot explain the results and that, whether or not the virus is solely endo-corpuscular or endo-leucocytic at some stage, it was present in the plasma in the cases examined.

Cases 10, 11, 13, 15 and 18 of the present series may be considered in this connection. For full details of the preparation of the materials injected we refer to the antecedent cases.

Cases 10 and 11 may be first considered together. Case 10 was injected with 1.3 c.c. of four-times washed corpuscles (see Case 4), and Case 11 with the corpuscle-free washings from the same case from blood taken at the same time. Both Cases 10 and 11 were definitely positive.

The 2 c.c. of washings in Case 4 would roughly correspond to the plasma obtained from 0.3 c.c. of the original blood. This dose is small. The amount of corpuscles injected into Case 10 on the other hand was rather large. These two cases seem to us rather to support the contention that the virus is free in the plasma. The large dose of corpuscles used, and the probably great difficulty of freeing such material from adherent virus, may be the explanation of the success with the corpuscles. We unfortunately had no further opportunity of checking these results by injecting more nearly equivalent doses of the two materials.

The other experiments all concern the presence of virus in the fluid element (serum or plasma), and tend to support the view that the virus is naturally free in the fluid element.

Case 18 was injected with "washings" from Case 11, with a positive result. The dose of washings (which showed some haemolysis) was 2.6 c.c., which would be equivalent to the plasma from 0.5 c.c. of the original blood.

Cases 13 and 15, although not specially designed for the purpose, support the finding of virus in the fluid part of the blood. In Case 13 we obtained a positive result after injection of 2.3 c.c. of filtered citrated plasma from Case 10. The dose corresponds approximately to the plasma from 0.23 c.c. of the original blood. Haemolysis was not noted. In Case 15, 2 c.c. of filtrate



from the serum of Case 10 produced a positive result. This serum filtrate was distinctly haemolysed and the dose (2 c.c., undiluted) was large.

#### THE INCUBATION PERIOD.

Our previous results showed that the incubation period of dengue varied between 5 and  $9\frac{1}{2}$  days. We found no incubation periods as short as those described by Ashburn and Craig(4), and by Graham(3). The present series confirms the view that the incubation period is usually about a week, but we have a greater variation in the periods than we encountered previously. However, our shortest period is still considerably longer than the incubation period found by these other observers in Syria and the Philippines.

The incubation periods observed in the successful cases were as follows: 8,  $6\frac{1}{2}$ , 15, 10, 9, 7, 8, 5, 7,  $4\frac{1}{2}$  days (in Case 17 the exact period could not be determined).

The shortest incubation period was actually about 4 days 19 hours (Case 18), and the longest almost exactly 15 days.

There does not seem to be any relation between the time the virus was outside the body and the incubation period, nor between the duration of illness of the previous case and the incubation period.

The cause of the variation is not known.

#### *Immunity.*

There is only one experiment bearing on immunity, namely, Case 3. In this instance the subject of the experiment was injected at the same time, with an approximately equal amount of the same material, as was used to inoculate Case 2 (a non-immune). Whereas Case 2 developed typical symptoms, Case 3 had at no time subsequent any signs of the disease.

Case 3 was the subject of mosquito experiments referred to in our earlier communication(1) and had passed through a typical attack of rather severe dengue starting on 31. v. 16. On 15. i. 17, at 4.15 p.m., Case 3 was inoculated subcutaneously with about 0.5 c.c. of diluted blood from Case 1, and thereafter showed no signs of the disease. Thus 7 months and 15 days (229 days) after the onset of an attack of dengue immunity was present to a dose of virus, which brought about a typical and rather severe result in the control case.

#### PART II. MOSQUITO EXPERIMENTS.

Although a number of experiments were attempted with various species of mosquitoes, *Stegomyia fasciata*, *Culex fatigans*, *Culicelsa vigilax*, *Culicelsa annulirostris* and *Scutomyia notascripta*, most of these were failures because the mosquitoes died out or would not bite, or because volunteers were not available. Special experiments with *S. fasciata*, hatched from larvae brought from Mullumbimby, similarly failed. The following experiments, however, are quoted in detail.



*With Culex fatigans.* 22 ♀♀ and 1 ♂ of this species were collected in Sydney and enclosed in a cage with a muslin sleeve. They were allowed to feed on Cases 10 and 11 on 15 and 16. II. 1917, and bit both patients well. Case 10 was thus bitten three or four days after the onset, and Case 11 five and six days after the onset. Case 10 was shown to have virus in the blood a few hours before the biting on 15. II. (see Case 15). Case 11 was bitten rather late and probably the blood at this time did not contain any virus. Many of the mosquitoes were alive on 18. II., but could not be counted owing to the nature of the box. On 18. II. and each night until 26. II. (inclusive) the mosquitoes remaining alive bit Volunteer Mrs W. G. A. No result followed. Although it is not possible to say exactly how many mosquitoes bit either the donor or recipient, it is certain that both were bitten by the mosquitoes in the cage.

The experiment tends to support previous experiments which failed to demonstrate that *Culex fatigans* is capable of transmitting dengue.

*With Culicelsa vigilax.* About 40 of this species were caught at Berowra on 15. II. 1917, and on the same day bit Case 10 (three days after the onset). Again on 16. II. (four days after the onset) at midday he was bitten. The mosquitoes also bit Case 11 on the same day (five days after the onset) and on 16. II. (six days after the onset).

There were only about a dozen mosquitoes left alive when on 17. II. they were fed on a volunteer, H. McD.—one of the insects bit. After this but few were alive and none bit although tested daily until 21. II. for periods of 30–45 minutes.

No results followed. The experiment is inconclusive.

*Summary.* Several mosquito experiments in this series have not therefore added to our previous knowledge, but the one satisfactory experiment with *Culex fatigans* tends to support the view we hold that this species is probably not a vector of the disease. The results previously published by us support the opinion of Bancroft (5) and show clearly that, whether or not *C. fatigans* may also play a part in Australia, *Stegomyia fasciata* is capable of spreading the infection of dengue.

### PART III. ANIMAL EXPERIMENTS.

#### *An Attempt to Transmit the Virus through a Guinea-pig to a Human Being with a Negative Result.*

On 13. II. (at 3 p.m.) blood was taken from Case 10 (22 hours after the onset). This blood was shown, by the successful injections of Cases 13 and 14, to contain the virus.

On 13. II. (afternoon) a guinea-pig, No. 3647, was injected subcutaneously with 0.25 c.c. of blood (serum and corpuscles). The animal remained well and on 21. II. (morning) it was bled to death, and at noon on the same day 2 c.c. of the blood (serum and corpuscles) were injected into Case 22, P. W. P.,

m., 71 years. His temperature and pulse were taken daily for four days, and four-hourly for 14 days.

On 25. II. at 10 a.m. (four days after the injection) the temperature reached 99° F., and was on the same level during part of the day of 26. II., but this was unaccompanied by any other signs or symptoms, and the temperature after this remained normal.

There is no evidence, therefore, of the survival of the dengue virus after 7½ days in a guinea-pig or of its multiplication in this animal.

#### OTHER ANIMAL EXPERIMENTS.

These need not be separately detailed as they were uniformly negative.

Guinea-pigs and rabbits were injected intraperitoneally and subcutaneously without result, and sections from the organs of some of these were examined both by iron-haematoxylin and eosin stained sections and by Levaditi's method, and showed no abnormality. Our object in using Levaditi's method was in the hope of demonstrating possible spirochaete-like organisms and was undertaken in view of the results obtained with epidemic jaundice in France.

#### APPENDIX

##### DETAILED REPORT OF THE CASES DEALT WITH IN THE SERIES OF INJECTION EXPERIMENTS.

**Case 1, Dr F. G. (naturally infected).** This case forms the starting-point of the series of experiments with which this report is concerned.

The patient was on a holiday in Molangool, near Bundaberg, Queensland, where he states that occasional cases of dengue fever were occurring, and where mosquitoes (*Stegomyia fasciata*) were biting freely in the daytime. He left Molangool on 5. I. 17, passing the night in Bundaberg and thence journeying to Sydney by train. He arrived in Sydney on the evening of 7. I. 17. Thus his last day in Queensland, and the last day on which he was exposed to *Stegomyia* bites, was on 6. I. 17. He became ill on 11. I. in the evening, therefore the incubation period must have been at least five days, and, assuming he was infected in the Molangool district, six days or more.

He kindly volunteered to allow blood to be taken for our experiments, and accordingly one of us went to see him on the 13. I. at 4 p.m., and took about 1 c.c. of blood from the median basilic vein.

This case was apparently typical and fairly severe. He had the usual symptoms and a marked rash, which was very obvious on 13. I. He had not taken his temperature regularly and only stopped in bed one day.

*Experimental material.* The blood taken on 13. I. 17, at 4 p.m., ca. 46 hours after the commencement of the illness, was kept on ice until 15. I. at 4 p.m., when it was used to inject Cases 2 and 3. There was about 1 c.c. of clot and serum in the test-tube. This was shaken up with about 2 c.c. of sterile normal saline solution, and about 1 c.c. of the fluid part, containing



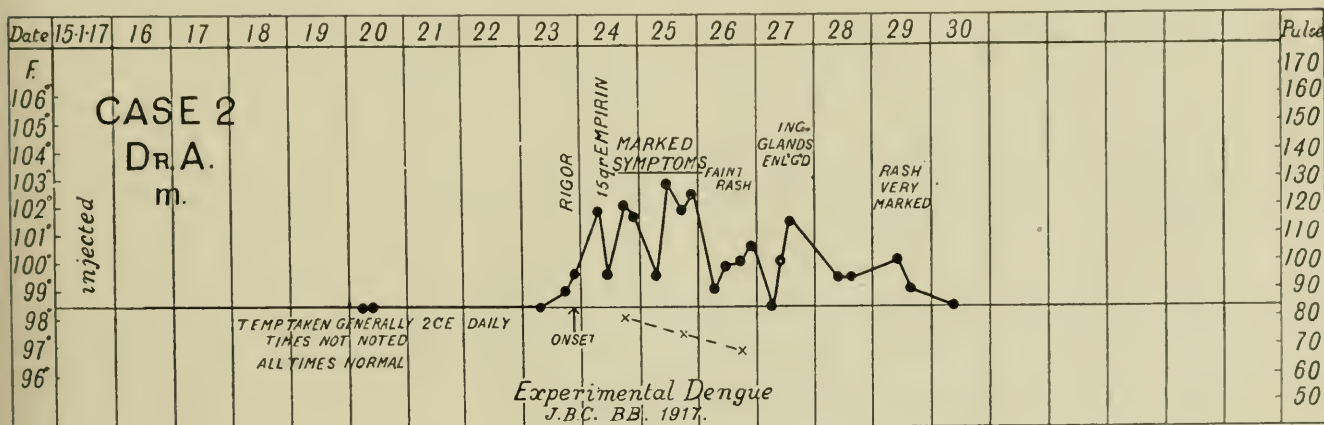
corpuseles, serum and saline, was sucked up into a syringe and equal amounts were injected into Cases 2 and 3:

Subcutaneous injection of 0.5 c.c. of diluted (1 to 2) blood taken from Case 1, 46 hours after the onset, virus kept outside the body 48 hours. *Result positive.*

**Case 2, Dr W. G. A., m.** 15. I. 17. On this date Dr A. of this Department volunteered for experiment, and at 4 p.m. he was injected subcutaneously with 0.5 c.c. of diluted blood from Case 1. The blood had been taken from Case 1 about 46 hours after the onset and had been outside the body for 48 hours; it was diluted ca. 1 to 2.

From this date the temperature, which was taken once at least and usually twice daily, remained normal until 23. I.

20. I. On this date he first felt slight rheumatic pains and some degree of languor on rising in the morning. The temperature was normal at 7 a.m.,



and at 9.30 a.m. There is no record of the evening temperature. After this there were occasionally some vague symptoms of languor, etc., but no rise of temperature until 23. I. Leucocytes 8500 per c.mm.

23. I. At 5 p.m. the temperature had risen to 99° F., and by 9 p.m. to 99.5° F. At 10 p.m. there was a rigor. A restless night was passed and he had occipital headache, painful eyes and vague rheumatic pains.

24. I. At 7.30 a.m. the temperature was 101.8° F. The patient came in to work and between rising and 11.30 a.m. took three 5-grain doses of aceto-salicylic acid. The temperature was 99.5° F. at 11.30 a.m. At midday he returned to his home. At 5.30 p.m. the temperature was 102° F. and the pulse 80, and at 9.30 p.m. the temperature was 101.6° F. At about 11 a.m. blood had been withdrawn from a vein and used to inject Cases 4 and 5. Blood from the ear and the serum from clotted blood from the vein were examined ultra-microscopically without finding any recognisable parasites.

25. I. He stopped in bed all day and had severe headache, pains in the limbs and eyes, and several chills. He took 5 grains of aceto-salicylic acid every three hours. The temperature at 7.30 a.m. was 99.5° F.; at 11.30 a.m.



102·8°; at 5.30 p.m., 101·8° (pulse 74); at 9.30 p.m., 102·4°. He passed a very restless night and did not sleep until 4.30 a.m.

26. I. A faint macular rash was discernible on the abdomen and thorax. The temperature ranged from 99° to 100·5° F. (see Chart).

27. I. The inguinal glands were distinctly enlarged on both sides. The temperature was 100° F. at 10.30 a.m., and 101·4° at 2.30 p.m. Leucocytes 3400 per c.mm. Blood was taken on this day about 11 a.m. from a vein and used to inoculate Case 6.

28. I. There was a faint but distinct morbilliform rash on the hands and forearms, most distinct on the palms. The temperature was 99·4° F. morning and evening.

29. I. The rash was very distinct and well-defined on the abdomen, chest, forearms, thighs, back, and palms of the hands. The face was free. The temperature was 99° to 100° F.

30. I. The temperature was normal and the patient felt practically well, although stiffness and a slight feeling of indisposition were present for some days afterwards.

*Remarks.* The case was a very definite one of dengue of rather severe type and the patient looked very ill for several days. The rash was extremely marked and very widespread. The incubation period, to the rise of temperature, was almost exactly eight days, but vague indisposition occurred just before five days had elapsed. Blood from this case, taken on the second day after the rise of temperature (24. I. 17), ca. 18 hours after the onset, gave a *positive result*, i.e. it induced the typical disease in Case 4, and blood taken on the fifth day of the disease (27. I. 17), or about 90 hours after the rise of temperature, caused the disease in Case 6.

*Experimental material.* (a) Blood was taken at 11 a.m. on 24. I. 17, ca. 18 hours after the onset as indicated by a rise of temperature. About 2·5 c.c. of blood was thus obtained and allowed to clot, and then ground up in a mortar with 2·5 c.c. of sterile normal saline solution.

1 c.c. of the more fluid part of this mixture was injected into Case 4.

1 c.c. of the same was injected into a guinea-pig, without result.

3 c.c. of the same was mixed with 2 c.c. of a saline emulsion of *B. coli communis* and filtered through a Pasteur-Chamberland F. candle and subsequently 10 c.c. of water were added. About 10 c.c. of red stained fluid came through fairly quickly. There remained in the filter candle about 5 c.c.

The filtrate was tested as follows:

On 24. I. 17, 1 c.c. was added to about 20 c.c. of broth. The original culture remained sterile up to 31. I., after which it was not further examined. Agar subcultures from this remained sterile. The last subculture was made on 31. I. and examined on 1. II.

On 24. I. 17 broth and agar cultures were made with one or two loops of the filtrate. These remained sterile until the last examination on 31. I.

The remaining filtrate (ca. 9 c.c.) was used to inject Case 5.

Although the above figures for dilution are only approximately accurate, it will be observed that the blood used to inject Case 4 was diluted ca. nine times with saline and water (1 to 9).

(b) Blood taken at 11 a.m. (90 hours after the onset) on 27. i. was not treated in any way but was used to inject Case 6.

Injection of a volunteer who had had dengue eight months previously, with virulent dengue blood. *Result negative.*

**Case 3, Dr B. B., m., 34 years.** One of us, who had been the subject of experimental mosquito-borne dengue eight months previously, was injected on 15. i. 17, at 4 p.m., with 0.5 c.c. of the diluted blood from Case 1. The amount injected was approximately equal to that injected at the same time into Case 2.

On 16 and 17. i. there was some headache, sore throat and languor but no areola round the injection and the temperature was normal. No symptoms developed after this date.

The result was *negative*, but Case 2, similarly injected, but not protected by a previous attack of dengue, developed this disease.

---

Subcutaneous injection of 1 c.c. of about equally diluted blood and saline solution taken from Case 2, 18 hours after the onset, virus kept outside the body  $3\frac{1}{2}$  hours. *Result positive.*

**Case 4, H. G., m., 40 years.** This volunteer was injected subcutaneously on 24. i. 17, at 2.30 p.m. with 1 c.c. of diluted blood serum and corpuscles from Case 2. The blood was taken from Case 2 at 11 a.m. on 24. i. 17 (18 hours after the onset). The dilution was with approximately equal parts of blood and sterile saline solution (for details see Case 2). The virus was outside the body about  $3\frac{1}{2}$  hours.

The patient remained well until 31. i. although he complained of slight headache a day or so before the definite onset.

31. i. 17. He arrived at the Laboratory looking very sick and said he had been taken ill about 3 a.m. He had pains "all over" and headache and felt very weak. His face was flushed. There was no definite rash. The temperature was  $100.4^{\circ}$  F. and the pulse 120, on arrival at the Laboratory about 10 a.m. The leucocytes were 8700 per c.mm.

Blood was taken on this day at about 11 a.m. from the median basilic vein and, after filtration, was used for the injection of Case 7, with a *negative result*.

2. ii. He stated that he had had two "bad" days at home, feeling sick, but was better now. There was a well-marked rash. Leucocytes 16,000 per c.mm.

Blood taken at midday from a vein was used as material to inject Cases 8—11, three of which were *positive*.



5. II. The patient felt fairly well.

Blood was taken at midday (?) and used to inject Case 12 with a *negative result*.

*Remarks.* The incubation period was  $6\frac{1}{2}$  days. Although a temperature chart was not regularly taken, the case was absolutely typical and moderately severe. The rash was well marked on the trunk and arms. Inoculation with blood taken from this case, 31 hours after the onset, gave a *positive result*, reproducing the disease in several cases.

*Experimental material. First specimen.* A specimen of blood was drawn from a vein on 31. I. 17, at about 11 a.m., about eight hours after the onset. A little serum was separated for the Bordet-Gengou test for syphilis, which proved to be negative.

The remaining serum and clot were mixed with about equal parts of boiled tap water. Some of this mixture was removed with the object of using it subsequently for injection but when required it was found to have become contaminated. The remainder was again equally diluted with a watery emulsion of a colon bacillus (*B. acidi lactici* type) and crushed up as far as possible in a mortar. The more fluid portion was put into the filter but would not pass through the candle. A further addition of water of approximately equal amount to the already diluted material was added. Filtration was slow and only about 2 c.c. of filtrate were obtained. The dilution, when filtered, was about 1 to 7, but this is not accurate having regard to the removal of the more fluid part for the Bordet-Gengou reaction, etc., and the final discarding of the clot.

Cultures were made from the filtrate—one and two loops on broth, and one and two loops on agar, and subsequently subcultures were made on agar from the broth. All cultures remained sterile.

The whole filtrate, consisting of about 2 c.c. of fluid, was injected into Case 7 with a *negative result*.

*Second specimen.* This was taken from a vein on 2. II. 17, at midday, ca. 57 hours after the onset.

(A) 2 c.c. were squirted from the syringe into ca. 10 c.c. of 1.5 % sodium citrate in normal saline. This was centrifuged for 15 minutes and the supernatant fluid separated from the deposit.

The *supernatant fluid* was then centrifuged for one hour and the fluid, down to  $\frac{1}{2}$  in. from the bottom of the tube, drawn off. This was labelled “*washings*,” and contained no corpuscles microscopically. The dilution would be 1 to 5. Cultures on broth and agar made on 2. II. remained sterile. These “*washings*” gave a *positive result* in Case 11.

The *corpuscles* from the first centrifugalisation were suspended in sterile saline solution and shaken and recentrifuged for 15 minutes. The supernatant fluid from this centrifugalisation was drawn off, fresh sterile saline added, and the mixture again shaken and recentrifuged for 15 minutes. The procedure was repeated and the residual corpuscles were retained. Cultures made on



agar and broth remained sterile. These four-times washed corpuscles were labelled "*washed corpuscles*." They gave a *positive result* in Case 10.

(B) About 5 c.c. of blood-clot were broken up with a sterile wire, and 2 c.c. of the more fluid part were separated and labelled "blood"; 0.8 c.c. (14 minims) of this were used to inject Case 8, with a *positive result*.

(C) The remaining 3 c.c. of clotted blood were diluted with about 9 c.c. of a watery emulsion of *B. coli* L.I.P.M., and were filtered in the usual way. The filtrate was tested by agar and broth cultures and found sterile. This was labelled "*filtrate*," and 2.8 c.c. were used to inject Case 9 with a *negative result*.

*Third specimen.* Blood was taken on 5. II. 17 at ? midday, and without dilution, 0.3 c.c. (5 minims) of serum and corpuscles were injected into Case 12. With *negative result*.

*Summary of experimental results obtained from material from Case 4.*

Diluted (1 to 7) filtered blood taken about eight hours after the onset gave a *negative result* (Case 7).

Undiluted blood taken 57 hours after the onset gave a *positive result* (Case 8).

Washed corpuscles taken 57 hours after the onset gave a *positive result* (Case 10).

Diluted (1 to 5) washings taken 57 hours after the onset gave a *positive result* (Case 11).

Diluted (1 to 3) filtered blood taken 57 hours after the onset gave a *negative result* (Case 9).

Undiluted blood taken approximately 130 hours after the onset gave a *negative result* (Case 12).

---

Subcutaneous injection of 9 c.c. of 10 times diluted filtrate (1 to 9) from Case 2, taken less than 18 hours after the onset. Virus kept outside the body ca. 29 hours or less. *Result negative*.

**Case 5, P. N., m.** On 25. I. 17, this volunteer was injected, at a time not noted but probably about 4.30 p.m., with 9 c.c. of a filtrate prepared from blood taken from Case 2 on 24. I. 17, at 11 a.m., *i.e.* ca. 18 hours after the onset. The virus was thus outside the body probably about 29 hours. This dilute filtrate would correspond roughly to the fluid from 0.9 c.c. of blood. The arm was sore and showed an erythematous flush for a few days around the site of inoculation, and the temperature rose next day to 99° F. This rise was evidently due to toxins in the material inoculated, probably from the colon bacilli used as a test for possible permeability of the filter.

Examination of the temperature chart (not published), which was kept from 28. I. until 28. II. 17, and was taken four-hourly from 3 to 28. II., shows a rise to 99.4° F. on the ninth day, followed by several minor rises

above normal, until on the 22nd day the temperature reached 100° F. followed by 99·2° F. on the 23rd and 24th days of illness.

During the whole period that this chart was kept, the patient worked hard and felt well. His body was examined daily but showed no rash or other signs of infection. The pulse was variable, being usually rather slow and often at about 50 and sometimes lower.

*Remarks.* Taking all things into consideration, although it is possible that there may have been a modified reaction to the virus, the case must, for the purposes of proof of filtrability of the dengue virus, be regarded as *negative*. This experiment is discussed separately in the section dealing with filtrability of the virus.

Subcutaneous injection of 0·5 c.c. of undiluted blood from Case 2, taken 90 hours after onset. Virus kept outside the body 6½ hours.

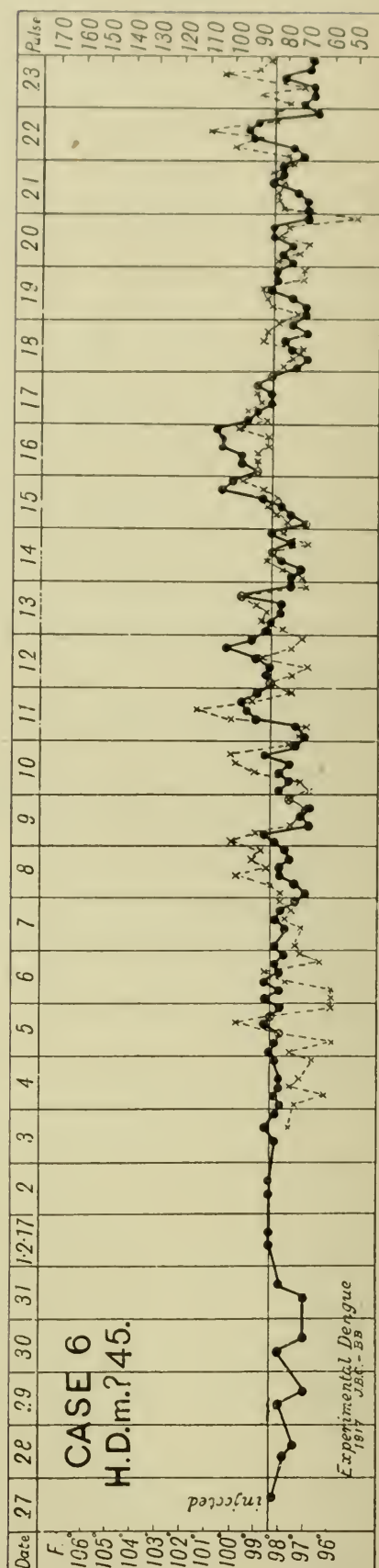
*Result positive.*

**Case 6, H. D., m., 45 years.** This case was injected at 5.30 p.m. on 27. I. 17 with 0·5 c.c. of untreated blood taken from Case 2 on 27. I. 17, at 11 a.m. The virus had been taken 90 hours after the onset and was outside the body 6½ hours.

This patient felt a little "out of sorts" on the afternoon of 11. II. 17, and had a rash on his shoulders with a temperature at 6 p.m. of 99·6° F.

12. II. 17. On examination the face was suffused, the eyes and pharynx injected, the tongue slightly coated, and a slight cough was present. There was a well-marked rash consisting of a pinkish raised papular erythematous mottling over the whole body, except below the knees and on the hands. The rash tended to run together into large patches. It was very copious in the armpits and groins. It began on the shoulders and quickly covered the upper three-fourths of the body.

13. II. The rash was more copious but





slightly duller in colour. The distribution was the same. The rash was seen by several medical men and photographed. The patient felt well.

14. II. The rash was fading. There was still some cough. The patient felt well.

15. II. The same as on 14. II.

16-17. II. The rash was only visible on the back. The patient felt well.

18-21. II. There was now only an erythematous blush on the back and no rash. The patient felt well.

23. II. The patient was allowed up.

*Remarks.* The chart of this case shows an irregular diphasic temperature variation with a late third rise. The incubation period, measured to the first definite rise of temperature, is about 15 days, which is double that of the usual period found in our first series of cases. However, the present series contains other instances of long periods and the case cannot be rejected as being positive on this account. The symptoms were very mild but the patient was of a very low mental type and it was hard to get intelligent replies to questions.

A specimen of blood was taken with the object of using it for further inoculations, but the case gave a strongly positive Bordet-Gengou ("compluetic") test and thus prevented this being done. The rash was in our opinion, and in that of others who saw it, definitely not a syphilitic manifestation and equally definitely was of the type seen in dengue. The case bore evidence of old, probably congenital, syphilis and no evidence of recent infection.

The temperature-pulse relationship, although not typical of dengue as seen in some of the charts, shows for the most part a relatively slow pulse in relation to the height of temperature.

---

Subcutaneous injection of 2 c.c. of filtered blood from Case 4, the blood taken eight hours after the onset in Case 4. Virus kept outside the body 30 hours. *Result negative.*

**Case 7, W. J. I., m., 72 years.** On 1. II. 17, at 4.30 p.m., the volunteer received subcutaneously 2 c.c. of the filtrate from Case 4. The blood from which this was obtained was taken on 31. I. 17, eight hours after the onset, and was kept outside the body for 30 hours. The temperature was charted for 22 days twice daily and, except for a rise to 99° F. on the day after the injection, remained normal.

*Remarks.* This case gave a definitely *negative result* with filtered diluted blood. There was no control case injected with untreated blood taken on this date, but blood taken on the third day of Case 4 gave *positive* results, and therefore it is almost certain that blood taken on the first day was infective and that the failure of the filtrate to produce infection cannot be attributed to an absence of virus in the blood.

This case is discussed fully in the section on filtrability of the virus.

---



Subcutaneous injection of 0.8 c.c. of untreated blood taken from Case 4, 57 hours after the onset. Virus kept outside the body 99 hours. *Result positive.*

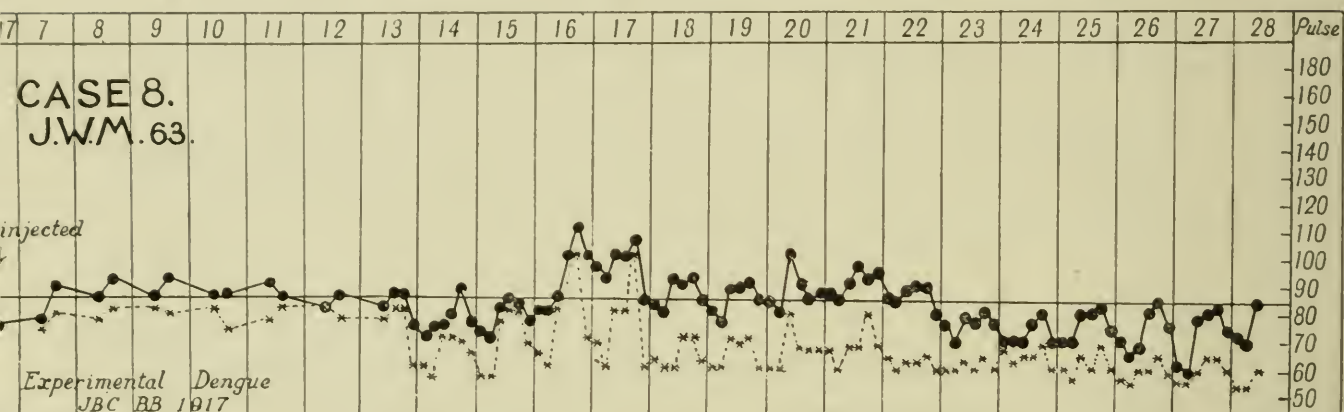
**Case 8, J. W. M., m., 63 years.** On 6. II. 17, at 3 p.m., the case received subcutaneously 0.8 c.c. of blood which had been withdrawn from Case 4 on 2. II. 17 at noon, that is, 57 hours after the onset, and kept on ice for 99 hours.

The temperature, as can be seen on the chart, was slightly above normal for several days after the injection, but between 11. II. and 15. II. it remained practically normal or subnormal.

16. II. When the patient entered the ward at 2 p.m. to have his temperature taken, he looked flushed, and his temperature was found to be 100° F., but he did not complain of any symptoms. That evening the temperature rose to 101° F.

17. II. He was examined in bed: face flushed, eyes clear, tongue not coated, pharynx injected, slight cough but no coryza. He stated he felt well. There was an erythematous flushing of the back. Maximum temperature 100.5° F.

18-19. II. No rash present, tongue coated. Temperature was 99.2° F.



20. II. Tongue clearing, a faint mottling on the abdomen and back. Maximum temperature 100.2° F.

21. II. Mottling on chest, back and abdomen, but not on legs and arms. The patient felt well. Temperature 99.7° F.

27. II. Patient allowed up. The rash was fading. Temperature had been subnormal since 23. II.

**Remarks.** The incubation period was ten days. The temperature variation was roughly diphasic having its maxima at 6 p.m. on 16. II. and at 10 a.m. on 20. II. The pulse tended to be slow and this bradycardia is noticed on the chart to start several days before the definite onset. During the first period of pyrexia the pulse was on two occasions raised correspondingly with the temperature, but for the most part and especially in the later stages of the pyrexia remained relatively slow, until the end of the temperature-taking.

Although slow, there was a noticeable correspondence between the oscillations of the pulse and those of the temperature, a correspondence which is seen in several cases. The most striking feature was the entire absence of symptoms. There can be little doubt, however, that this case was one of mild dengue infection.

The patient gave a partially positive Bordet-Gengou (compluetic) reaction, and therefore further inoculations could not be practised.

Subcutaneous injection of blood filtrate from Case 4. The blood was taken 57 hours after the onset and was kept outside the body 28 hours. *Result negative.*

**Case 9, J. W., m., 65 years.** On 3. II. 17, at 4.30 p.m., 2.8 c.c. of filtrate (diluted 1 to 3) of blood from Case 4 taken on 2. II. (noon), 57 hours after the onset, were injected subcutaneously. The virus had then been outside the body for 28 hours.

The temperature was taken twice daily for seven days and thereafter four-hourly for 14 days. There was a rise to 99.4° F. on 7. II., and there were slight oscillations later not exceeding 99°, but insufficient to indicate infection. There were no symptoms, the patient feeling perfectly well during the whole period.

*Remarks.* Result negative. There were three other cases (8, 10, 11) inoculated with unfiltered preparations of blood taken at the same time, all of which were *positive*.

The case is fully discussed under the section dealing with filtrability.

---

Subcutaneous inoculation with 1.3 c.c. of washed corpuscles from Case 4.

The blood was taken 57 hours after the onset, and was kept outside the body 28 hours. *Result positive.*

**Case 10, P. M., m., 45 years.** On 3. II. 17, at 4.30 p.m., 1.3 c.c. of washed corpuscles from Case 4 (*q.v.*) were injected subcutaneously into this case. The blood from which the corpuscles were derived had been taken from Case 4 on 2. II. 17, at noon, that is, 57 hours after the onset. The virus was kept outside the body 28 hours.

12. II. 17. At 5 p.m. he became suddenly ill with severe frontal headache and flushing. During this night he felt hot, flushed and headachy. The temperature rose steeply in the evening, reaching 101° F. at midnight.

13. II. Face flushed, eyes injected, tongue coated, no coryza. He stated that the headache had now practically gone. There was an erythematous flushing of the back but no rash. Temperature varied between 100° and 101° F. Blood was taken for experimental purposes. The Bordet-Gengou (compluetic) test was negative.

14. II. Face still flushed, eyes injected, tongue coated. He felt well. A faint mottling on the back. Temperature varied between 100° and 101° F.



15. II. Temperature lower, the highest point reached being 100° F. There was still faint mottling on the back. The patient felt well. Another sample of blood was taken.

16. II. Maximum temperature 100.2° F. Mottling still visible on the back. The patient felt well.

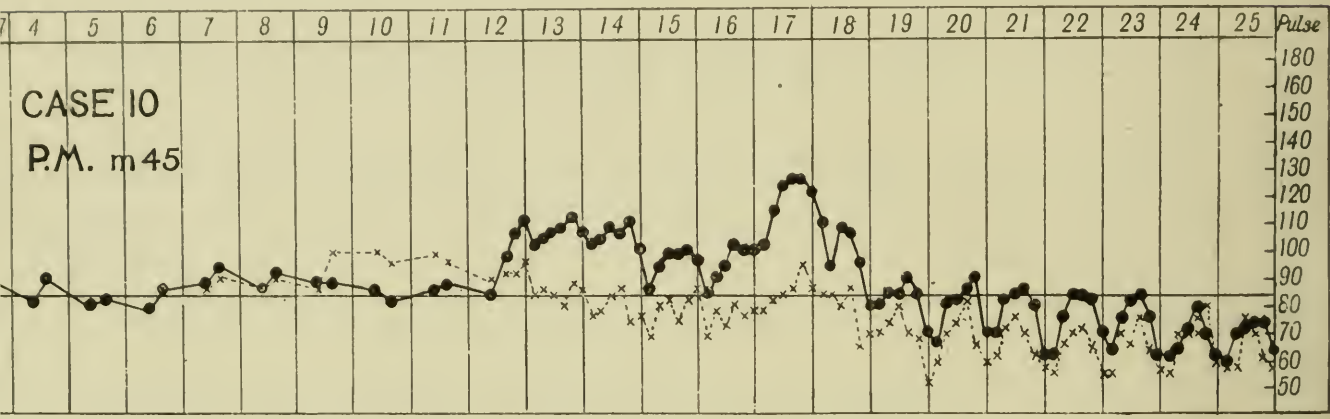
17. II. Rash still present on back and faintly marked on chest. He complained of feeling hot. Temperature rose to 102.8° F.

18-19. II. He did not feel so well. Temperature fell from 102° F. to subnormal.

20. II. He felt well. Maximum temperature 99° F. A third specimen of blood was taken.

After this the temperature did not rise above 99° F., and after 21. II. 17 was normal or subnormal. He was allowed up on 22. II. The rash was still present but he felt well.

Remarks. The inoculation undoubtedly caused a definite attack of dengue. The incubation period was nine days. The temperature variation



was definitely diphasic of the saddle back type. The pulse showed definitely the failure to respond proportionately to a rise in temperature so frequent in these cases. There was relative bradycardia from the beginning to the end of the period of pyrexia, and after the attack, the pulse was periodically absolutely slow. In this case we note again a rough correspondence in the chart between the oscillation of the pulse and that of the temperature, although the pulse is throughout the illness slow.

It will be noted that prior to the onset there was a period during which the pulse rate was higher than usual, but as soon as the pyrexia appeared the pulse became slower, only rising during the second part of the pyrexia to 95, although the temperature was 102.8° F.

Blood from this case, taken 22 hours and 67 hours after the onset, reproduced the disease, but blood taken eight days (ca. 190 hours) after the onset gave a negative result.

Experimental material. First specimen. Blood was withdrawn on 13. II. 17, at ca. 3 p.m., i.e. ca. 22 hours after the onset.



(a) 2 c.c. of this was squirted into about 8 c.c. of sterile 1.5 % sodium citrate in normal saline solution. This mixture was centrifuged free from corpuscles and filtered in the usual way after adding scrapings of an agar culture of *B. coli*. Filtration was rapid, ca. 5 c.c. of fluid being obtained in a few minutes. The dilution was 1 to 4.

Cultures were made by adding two loopful of filtrate to 10 c.c. of broth and also by smearing a loopful on agar. These cultures remained sterile.

2.3 c.c. of the filtrate were used to inject Case 13 on 15. II. 17 at 4 p.m. with *positive result*.

(b) Serum from the clotted blood gave a negative Bordet-Gengou (complementary) reaction.

Accidentally most of the clotted blood was thrown away, but there were a little serum and corpuscles left in the test-tube and these, after being shown to be sterile by culture, were injected into Case 14 on 15. II. 17 at 4 p.m. About 0.3 c.c. of blood was injected with *positive result*.

*Second specimen.* A large sample of blood, about 20 c.c., was withdrawn on 15. II. 17, at about midday, ca. 67 hours after the onset.

About 10 c.c. of serum were freed from corpuscles by centrifugalisation; scrapings of a culture of *Staphylococcus aureus* were added and the material filtered. Filtration was rapid. Cultures on broth (two loops) and agar (one loop) were sterile. 2 c.c. of the filtrate were used to inject Case 15 with a *positive result*.

*Third specimen.* Blood was taken on 20. II. 17, allowed to clot, and 1 c.c. of serum and corpuscles were used that day to inject Case 16, with *negative result*. This specimen was taken from Case 10 about 190 hours after the onset.

---

Subcutaneous injection of 2 c.c. of "washings" from Case 4, taken 57 hours after the onset. The material was kept outside the body for 28 hours.

*Result positive.*

**Case 11, J. D., m., 74 years.** On 3. II. 17, at 4.30 p.m., J. D. was injected subcutaneously with 2 c.c. of (1 to 5 diluted) corpuscle-free washings from the citrated blood of Case 4, the blood was taken on 2. II. at noon, 57 hours after the onset, the virus having been kept outside the body for 28 hours.

8. II. He complained of languor and some diarrhoea. Temperature normal.

9. II. He said he felt better but was "tired." Temperature normal.

10. II. Temperature rose to 99.0° F. at 4.25 p.m. During the night he felt so giddy and headachy that he was put to bed. (He was employed as night-watchman.) Temperature rose to 101.6° F. by midnight.

11. II. Temperature at 4 a.m. 102.4° F., ca. 101° to 102° during the day.

12. II. Face flushed, eyes and pharynx injected, tongue coated. There was pain in the neck and lumbar region and slight cough but no coryza. There was no rash. The patient complained of sleeplessness. Blood was taken on

this day and gave a *positive* result in the form of a filtrate in Case 17 and as “washings” in Case 18.

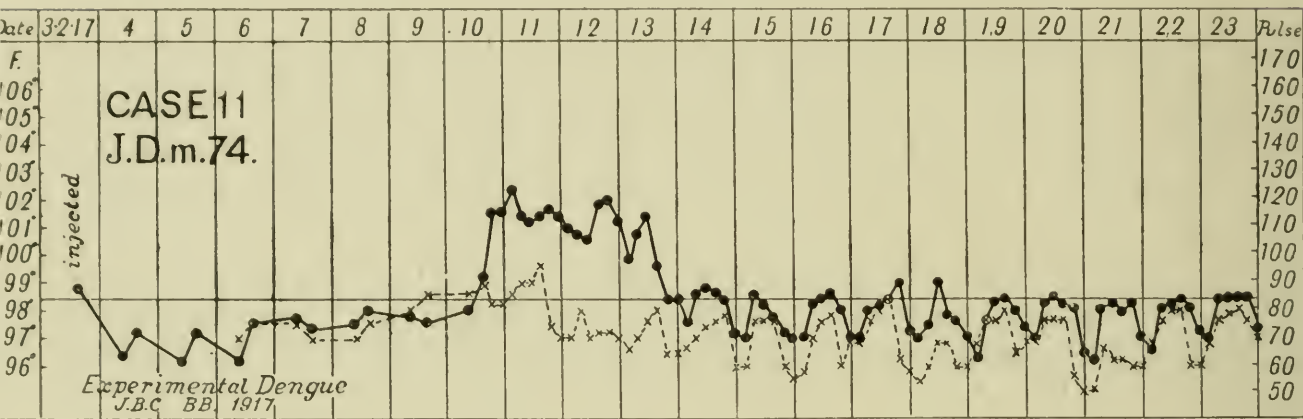
13. II. He felt much better and there were no pains. There was some suggestion of a rash. Temperature fell to normal.

14. II. A pinkish erythematous mottling over the whole back, chest, abdomen, thighs, shoulders and arms, but nothing on the legs, feet or hands. He felt well. Temperature about normal.

15. II. The rash fading. Blood taken on this date gave a *negative* result as a serum filtrate injected into Case 19. The temperature was normal at this time.

16–22. II. The rash faded gradually and the patient felt quite well. He was allowed up on 22. II. There were rises of temperature on 17 and 18. II. to 99° F.

26. II. He complained of weakness. The temperature had been normal or subnormal since 18. II.



*Remarks.* The incubation period, measured to the first rise of temperature, was seven days. The previous symptoms are regarded as premonitory. The chart shows a definitely maintained rise of temperature from 10 to 13. II. inclusive. On the evening of 13. II. the temperature fell sharply and thereafter, except for two isolated rises to 99° F. on 17 and 18. II., was normal or subnormal until 23. II. (end of chart).

The pulse-temperature relationship was “typical.” Except for a slight increase on the day after the onset of fever, the pulse failed to respond to the rise of temperature. Relative bradycardia is marked. Towards the end of the chart are seen occasional periods of definite absolute bradycardia. Although the rash was not marked it was definite. *The result was positive.*

*Experimental material. First specimen.* On 12. II. at 3 p.m., 47 hours after the onset, about 10 c.c. of blood were taken from a vein. 7 c.c. of this were allowed to clot—about 1 c.c. of the serum was then abstracted for a Bordet-Gengou (compluetic) test (which proved negative), and also another 1 c.c. of serum and corpuscles was abstracted for injection purposes but not used. The remaining serum and clot (about 5 c.c.) were mixed with about



10 c.c. of a watery emulsion of colon bacilli and shaken well together. After allowing the solid material to deposit, the more fluid part of the mixture was again equally diluted with *tap-water* and filtered in the usual manner through a Pasteur-Chamberland F. filter.

About 5 c.c. of filtrate were rapidly obtained (the candle still retained about 6 c.c. of material). The filtrate was tested by making cultures on agar (one loop) and on broth (two loops), and these remained sterile. The specimen was labelled "blood filtrate, Case 11," and about 2 c.c. were used to inject Case 17 with a *positive result*. The dilution was approximately 1 to 5.

About 2 c.c. of the original blood was mixed with about 5 c.c. of 1·5 % sodium citrate normal saline solution. The mixture was centrifuged for half-an-hour and the supernatant fluid removed. Some haemolysis had occurred and the supernatant fluid still contained some red corpuscles. The supernatant fluid was recentrifuged, poured off, and left overnight. On the next day this was again recentrifuged and the supernatant fluid removed and labelled "washings from Case 11." 2·6 c.c. were used to inject Case 18 with a *positive result*.

We have no note as to the microscopical examination of the final fluid but it is safe to say it was then free from corpuscles. The dilution was approximately 1 to  $2\frac{1}{2}$ .

*Second specimen.* About 15 c.c. of blood were taken on 15. II. 17 at about noon, and the serum was centrifuged free from corpuscles. About 3 c.c. of the serum, distinctly stained with haemoglobin, were obtained and this was diluted equally with water. To the dilute material a loopful of an agar culture of *Staphylococcus aureus* was added. It was filtered in the usual way through a Pasteur-Chamberland F. candle. The filtrate came through rapidly and was labelled "*serum filtrate from Case 11.*" Cultures made on agar (one loop) and broth (two loops) proved sterile. This filtrate was used to inject Case 19 with a *negative result*.

---

Subcutaneous injection of 0·3 c.c. (5 minims) of untreated blood from Case 4, ca. 130 hours after the onset. Virus kept outside the body less than 24 hours. *Result negative.*

**Case 12, P. B., m., 61 years.** On 6. II. 17, at 10 a.m., he received subcutaneously 0·3 c.c. of serum and corpuscles from Case 4, which had been taken on 5. II. about 130 hours after the onset. The virus was kept outside the body for less than 24 hours.

The temperature and pulse were taken twice daily for six days, and then every four hours for 16 days, but no indications of infection followed.

*Remarks.* The injection gave a *negative result*, this indicating that the virus was not present in the blood of Case 4 on the sixth day (ca. 130 hours) from the onset.



Subcutaneous injection of 2.3 c.c. of citrated plasma filtrate from Case 10.

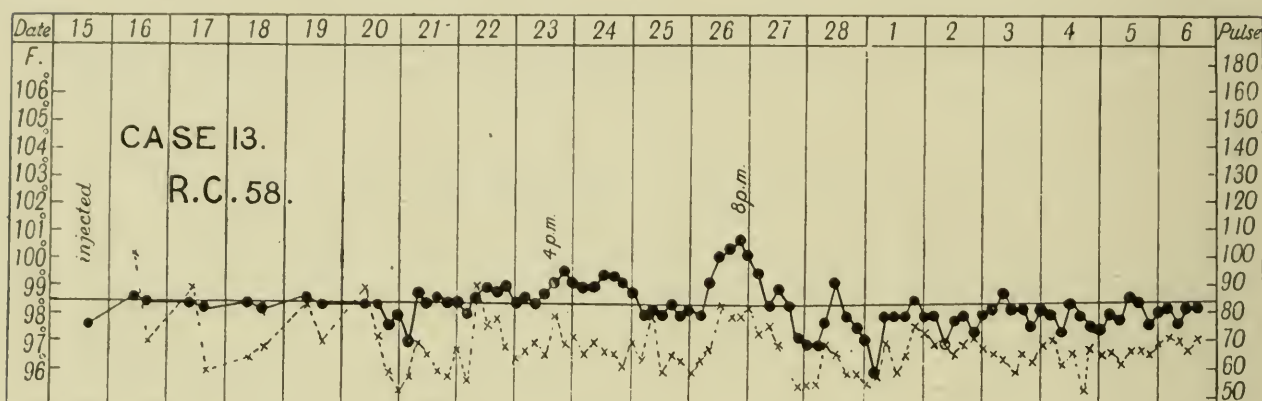
The blood was taken about 22 hours after the onset. Virus kept outside the body 59 hours. *Result positive (mild).*

**Case 13, R. C., m., 58 years.** On 15. II. 17, at 4 p.m., R. C. was injected subcutaneously with 2.3 c.c. of citrated plasma filtrate taken from Case 10. The blood was withdrawn from Case 10 at 3 p.m. on 13. II. or 22 hours after the onset. The virus was kept outside the body for 48 hours.

21-22. II. The temperature was as high as 98.8° F. at 8 a.m. on 21. II. but thereafter remained about normal until midday on 22. II., when it reached 99° F.

23. II. Temperature was 99.2° F. at 4 p.m., and 99.6° at 8 p.m. The patient complained of feeling "out of sorts," and was put to bed for observation.

24. II. Temperature remained at 99° F. during the day, rising to 99.4° in the evening. He had slight headache and depression and also a faint flushing of the face and back.



25. II. Temperature fell and the patient felt well.

26. II. Patient complained of headache during the previous night. Tongue coated, face and back flushed, but there was no rash. During the afternoon and evening, the temperature rose rapidly, reaching 100.8° F. at 8 p.m.

27. II. Temperature subnormal during the morning, and 99° F. at midday.

28. II. Since 28. II. the temperature had been normal. On this date the patient complained of pain in the chest and was not so well.

3. III. Slight headache and flushing of the back were present. Tongue coated.

There was nothing further to note after this date, and the patient got up on 5. III.

*Remarks.* It is hard to fix the time of onset of the pyrexia, as will be seen by reference to the chart. As early as 21. II. there was a rise to nearly 99° F. and during 22. II. the temperature was on the 99° level. On 23. II. the temperature rose definitely, reaching 99.6° F., and on 24. II. the highest point was 99.5° F. On the evening of 26. II. there was a steep rise to 100.8° F.

at 8 p.m., but by 8 a.m. on 27. II. this had subsided to rise to  $99^{\circ}$  at midday. Thereafter, with the exception of one slight rise to  $99.2^{\circ}$  on 28. II. the temperature was practically normal.

Considering the occurrence of symptoms, mild but definite, on the evening of 23. II., it is probable that the onset occurred about this time and was followed by the mild attack of dengue. Assuming that the evening of 23. II. represents the beginning of the attack, the incubation period was eight days. The type of chart was definitely diphasic with an initial slight pyrexia, and a secondary more definite pyrexia on 26. II.

The pulse is characteristic in both phases, failing almost completely to respond to the rise of temperature.

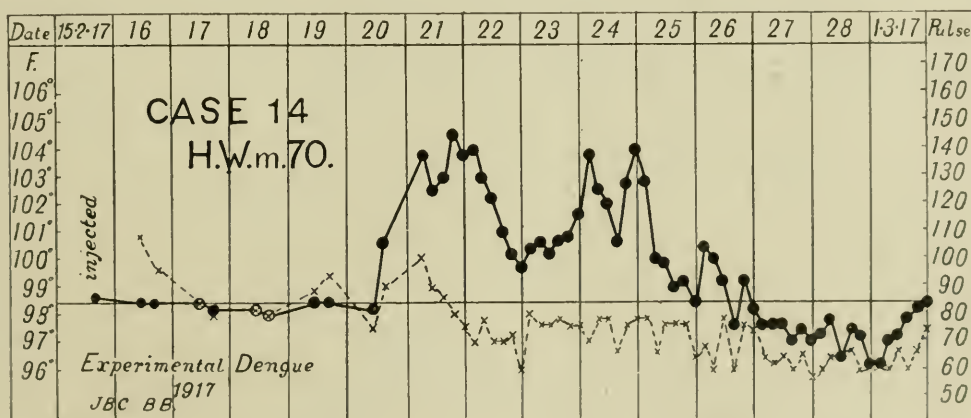
*Experimental material.* It was impossible to utilise the blood from this case for further experiments as there were no volunteers obtainable.

Subcutaneous injection of about 0.3 c.c. of untreated blood (serum and corpuscles) taken from Case 10, about 22 hours after the onset. Virus kept outside the body 49 hours. *Result positive.*

**Case 14, H. W., m., 70 years.** On 15. II. 17, at 4 p.m., H. W. was injected with about 0.3 c.c. of serum and corpuscles taken from Case 10 on 13. II. at 3 p.m., *i.e.* 22 hours after the onset. The virus was kept outside the body for 49 hours.

20. II. Temperature  $100.6^{\circ}$  F. at 3 p.m. Patient said he felt "flushed" but he had no other symptoms.

21. II. Temperature  $103.8^{\circ}$  F. in the morning, and  $104.6^{\circ}$  in the evening, but the patient said he felt well. Face flushed, eyes injected, tongue coated. A distinct erythematous flushing of the back but no definite rash.



22-23. II. A faint mottling on the back but nothing else to note. Patient felt well. A distinct fall of temperature occurred to  $100^{\circ}$  F. but it gradually rose again.

24. II. Patient said he did not feel so well. Temperature rose sharply to  $103.8^{\circ}$  F. in the early hours of the morning, and fell during the day to  $100.6^{\circ}$  F., rising again at night to  $104^{\circ}$ .



25. II. Temperature fell rapidly to 99° F. The patient felt well.

26. II. Temperature rose to 100·4° F., but fell again to subnormal, rising again at 8 p.m. to 99·2°.

27. II. Temperature subnormal and remained normal or subnormal afterwards until 2. III. Patient allowed up on 1. III.

*Remarks.* The incubation period is about five days. The chart is perhaps the most typical of the series. It shows a distinct diphasic variation with maximum points of 104° F. and over, and a distinct remittent period.

The pulse was absolutely characteristic. It rose slightly with the first rise of temperature but thereafter fell and remained normal during the subsequent pyrexia. The occurrence of a temperature of nearly 105° F. with a pulse rate of 80 needs no further comment.

The extraordinary absence of symptoms was most impressive and is a striking instance of a pathological process affecting the thermo-regulatory system without obviously affecting any other.

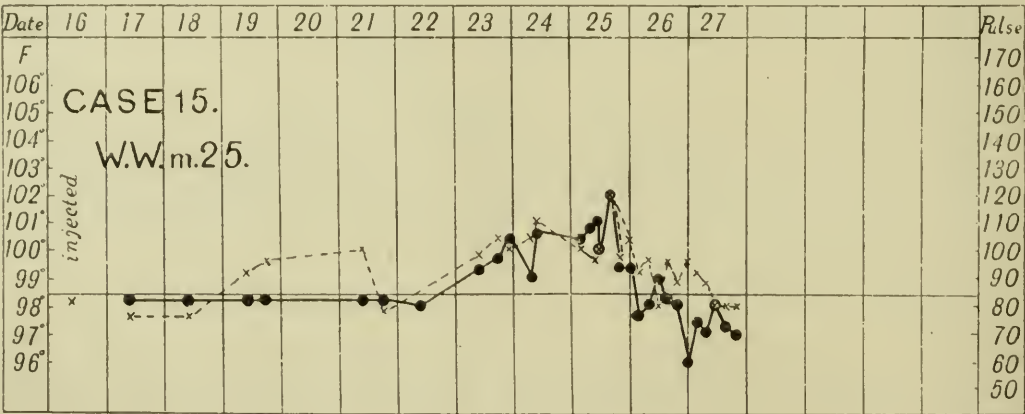
*Experimental material.* Although blood was twice taken with a view of using it for further experiments, the absence of volunteers prevented us making any use of the material.

Subcutaneous injection of 2 c.c. of undiluted serum filtrate from Case 10.

The blood was taken 67 hours after the onset. Virus kept outside the body 22 hours. *Result positive.*

**Case 15,** *W. W., m., 25 years.* On 16. II. 17, at 10 a.m., this case was injected subcutaneously with 2 c.c. of a “serum filtrate” from Case 10. The blood was taken from Case 10 on 15. II. at noon (67 hours after the onset). The temperature was normal until 23. II.

23. II. The patient became ill with typical and fairly severe symptoms. Malaise, bad headache and body pains were present. He continued to work. His temperature rose from 99·3° F. in the morning to 100·4° at 10 p.m.



24. II. Temperature at 7.30 a.m. 99° F. and at 9 a.m. 100·6°; it was not taken later that day. He was still going about his work but felt very sick. General pains were marked. There was pain in the eyes. He had a distinct



red mottled punctiform rash on the back, chest and buttocks, but not on the legs and arms.

25. II. Temperature rose to 102° F., but fell rapidly during the night. This day he remained in bed and felt very sick. Malaise and general pains were severe.

26. II. Maximum temperature 99° F.

27. II. Temperature was subnormal. He felt better and resumed ordinary duties. No further notes were taken but the rash was visible for several days and the patient complained of tiredness.

*Remarks.* The incubation period was seven days. This case was absolutely typical in symptoms and rash. The temperature variation is apparently monophasic. The pulse is not typical but it should be noted that this case had a tendency to a high pulse and was of a distinctly nervous temperament.

The blood from this case injected into Case 20 gave a *doubtful result*.

*Experimental material.* Blood was taken on 24. II. (24 hours after the onset), and 1.2 c.c. of the serum and corpuscles were injected into Case 20 on 27. II.

15 days later, Case 20 became febrile. His illness was most atypical, and cannot be considered definitely as dengue, especially as an attempt at further passage failed.

Subcutaneous injection of 1 c.c. of serum, containing corpuscles, taken from Case 10 on the eighth day of illness (about 190 hours after the onset).

The virus was kept outside the body at most a few hours. *Result negative.*

**Case 16,** *A. A., m., ? age.* On 20. II. 17, blood was taken from Case 10 and allowed to clot, and 1 c.c. of the serum was injected into this case at 5 p.m. The blood was taken eight days (190 hours) after the onset of Case 10. The virus was kept outside the body at most a few hours.

The chart was taken twice daily until 27. II., and thereafter every four hours until 13. III., and showed nothing to indicate any reaction to the injection.

*Remarks.* A *negative* result was obtained with serum from blood taken from Case 10 about 190 hours after the onset.

Subcutaneous injection of about 2 c.c. of blood filtrate from Case 11. The blood was taken 47 hours after the onset. Virus kept outside the body two days. *Result positive.*

**Case 17,** *N. K., f., 28 years.* On 14. II. 17, this patient received about 2 c.c. of a blood filtrate from Case 11 (*q.v.*). This had been prepared from blood drawn on 12. II. at 3 p.m. (47 hours after the onset of Case 11).

We have not a detailed history of this case as the circumstances of the volunteer did not permit us to take a chart, and when she became ill it was difficult to observe her frequently. The temperature was taken once daily

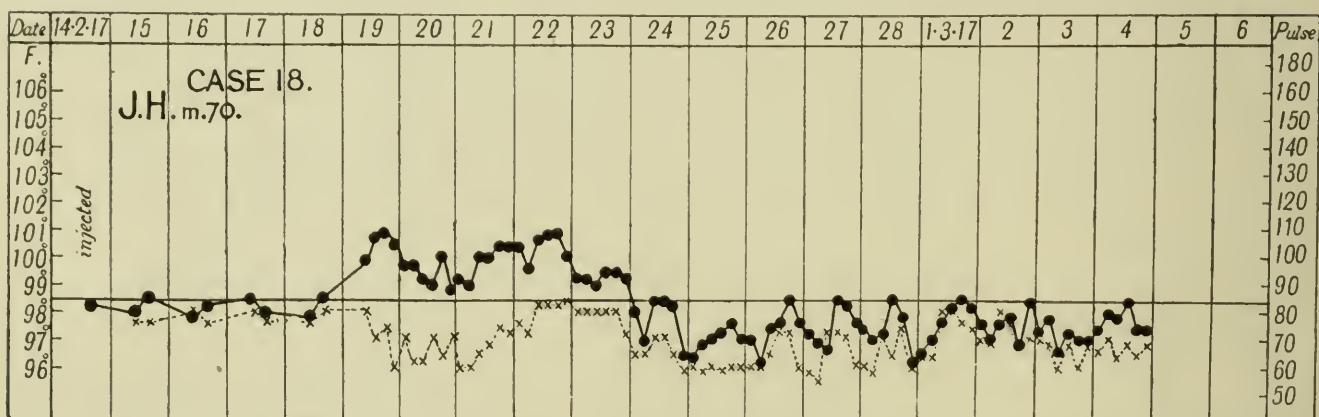
until 21. 11. and was always normal and the pulse between 76 and 86. It was impossible to fix the onset as the patient did not say anything about feeling ill until we observed the rash, and then the history was not definite as to dates. But on 26. 11. we observed that she had a very distinct rash, and on taking her temperature on the evening of that day it was found to be  $100.3^{\circ}\text{F.}$ , with a pulse of 100, and the patient had the typical appearance of a dengue patient with pains, headache, lassitude, etc., and showed a bright typical rash on the face, forearms, chest and back (further examination was not made). The girl was obviously ill for some days after this, but refused to go to bed and was extremely anxious that her employers should not know that she had dengue, so further investigation was not pressed. There was no doubt that her case was very typical, the rash being one of the best of the series.

*Remarks.* Typical moderately severe dengue with well-marked rash.

Subcutaneous injection of 2.6 c.c. of "washings" from Case 11. The virus was taken from Case 11 47 hours after the onset, and was kept outside the body 48 hours.

**Case 18, J. F. H., m., 70 years.** On 14. 11. 17, at 3 p.m., the case was injected subcutaneously with 2.6 c.c. of "washings" from Case 11 (*q.v.*). The blood was drawn from Case 11 on 12. 11. at 3 p.m., 47 hours after the onset. The virus was kept outside the body 48 hours.

18. 11. The patient complained of severe headache and pain in the chest on the afternoon of this date, but the temperature was normal.



19. 11. At 10 a.m. the temperature was  $99.8^{\circ}\text{F.}$  and the pulse 80. Face flushed, eyes injected, tongue coated. There was severe headache. There was no rash but the back was erythematous. At 6 p.m. the temperature was  $100.8^{\circ}\text{F.}$  and the pulse 74. The patient was sleepless at night.

20. 11. Headache still severe. Aspirin was given. There was no rash. The temperature was lower during the day ( $99.6^{\circ}\text{F.}$ ), but at 6 p.m. it rose to  $100^{\circ}$ , falling at midnight to  $98.8^{\circ}$ . He had a sleepless night.

21. 11. There was nausea, pain in the chest, and headache requiring



aspirin. There was no rash. In the morning the temperature was 99° F., but in the evening rose to 100·4°.

22. II. Headache better. He had slept well during the night, Temperature reached 100·8° F., but fell during the night. Pulse for the first time rose somewhat, reaching 84 at 10 p.m.

23. II. He had still slight headache and anorexia, but felt better. Temperature not above 99·4° F. There was still no rash.

24. II. He complained of weakness and anorexia. The tongue was coated. Temperature normal or subnormal on this date and subsequently.

*Remarks.* The incubation period was between 4 and 5 days (about 4 days 19 hours to the first definite pyrexia). This is shorter than the usual incubation period. The temperature variation was diphasic with high points on 19 and 22. II.

The pulse was relatively slow throughout the pyrexial period. It was especially slow during the first phase of the fever.

---

Subcutaneous injection of 2·3 c.c. of dilute serum filtrate from Case 11. The blood was taken from Case 11, 115 hours after the onset, and was kept outside the body 70 hours. *Result negative.*

**Case 19, T. B., m., 55 years.** On 18. II. 17, at 10 a.m., Case 19 received subcutaneously 2·3 c.c. of a (1 to 1) diluted filtrate from the serum of Case 11 (*q.v.*). The blood was drawn from this case on 15. II. at about noon, about 115 hours after the onset. The virus, if present, would have been outside the body for 70 hours.

The temperature and pulse were taken twice daily for ten days, then every four hours for 13 days. The temperature reached 98·8° F. on 21. II., but thereafter was normal or subnormal.

*Remarks.* The late stage, at which the blood was taken from Case 11, by itself is enough to explain the *negative* result.

---

Subcutaneous injection of 1·2 c.c. of untreated blood from Case 15, taken 24 hours after the onset, and kept outside the body about 72 hours. *Result doubtful.*

**Case 20, C. L., m., 70 years.** On 27. II. 17, at 7 p.m., the case was injected subcutaneously with 1·2 c.c. of blood from Case 15, taken on 24. II. (about 24 hours after the onset). The virus was kept outside the body about 72 hours.

The temperature and pulse were taken twice daily for five days and then every four hours for three weeks. There was a rise to 99·2° F. on 5. III., about six days after the injection, but thereafter the temperature was practically normal. On 13. III. the temperature reached 99°, and on 14. III., 15 days after the injection, it rose to 100·2°, then fell rapidly, rose next day to 99·8°, fell again, and rose next day to 100°, falling rapidly again. The following day the maximum was 99°, and thereafter the chart was normal until 25. III., when the patient was discharged.



*Symptoms.* On 6. III. he developed a mild attack of herpes. On 7. III., although the temperature was only 98·8° F. at its maximum, there was some flushing of the face and back, and slight coating of the tongue. After this until 14. III. nothing was noted. Examined on this date the patient stated that he became shivery the previous evening at 7 p.m. He had some slight headache, some injection of the eyes, no coryza and slight cough without expectoration. On 15. III. there was an erythematous flushing of the back, no rash and a slight cough. On 16. III. he complained of nausea but otherwise remained well. On 17. III. he stated that he sweated every night. The tongue was slightly coated. Nothing else was noted after this.

There was relative bradycardia with the pyrexia on 14, 15 and 16. III., but no absolute bradycardia.

*Remarks.* The case cannot be definitely regarded as dengue, nor can it be stated that it was not.

In view of the failure to transmit infection from this case to Case 21, and the atypical nature and long incubation period, it is best to regard the case as *doubtful*.

*Experimental material.* Blood, taken from this case on 14. III., on the second (?) day of illness but the fifteenth after inoculation, failed to convey infection to Case 21.

---

Subcutaneous injection of 2 c.c. of untreated blood from Case 20, taken on the second (?) day of illness, and kept outside the body about 48 hours.

*Result negative.*

**Case 21, H. McD., m., 42 years.** On 16. III. 17, at 4.30 p.m., this patient was injected with 2 c.c. of blood taken on 14. III. from Case 20. He had thereafter no symptoms of dengue and no pyrexia, although observed daily for several weeks.

---

Subcutaneous injection of 2 c.c. of serum and corpuscles from a guinea-pig injected 7½ days before with blood from Case 10, taken 22 hours after the onset.

**Case 22, P. W. P., m., 71 years.** The details of this case are found under Part III, Animal Experiments (bottom of p. 233).

#### REFERENCES.

- (1) CLELAND, BRADLEY and MACDONALD (2. IX. 1916). On the Transmission of Australian Dengue by the Mosquito *Stegomyia fasciata*. *Med. Journ. of Australia*.
- (2) — (5. II. 1918). Dengue Fever in Australia. *Journ. of Hyg.* XVI. 317-418, 9 charts; also in *Seventh Report of the Microbiological Laboratory of the Dept. of Public Health, N.S.W.*
- (3) GRAHAM (1. VII. 1903). The Dengue; A Study of its Pathology and Mode of Propagation. *Journ. of Trop. Med.* VI. p. 209.
- (4) ASHBURN and CRAIG (1907). Experimental investigations regarding the Aetiology of Dengue Fever. *Philipp. Journ. of Sc.* II. 93.
- (5) BANCROFT (I. 1906). On the Aetiology of Dengue Fever. *Aust. Med. Gaz.* p. 17.

## TWO INSTANCES OF HUMAN SERA SHOWING ABNORMAL ANTI-COMPLEMENTARY POWER.

BY E. L. KENNAWAY, M.D., D.Sc., AND CAPT. A. M. WRIGHT, N.Z.M.C.

(*From the Bland-Sutton Institute of Pathology, Middlesex Hospital, and V.D. Sect., No. 3 New Zealand General Hospital.*)

IN the great majority of cases human sera which have been heated at 55–57° C. for thirty minutes, as is the usual practice preparatory to the Wassermann test, exhibit only very slight anti-complementary properties. That is to say, complete haemolysis of the test corpuscles (0.5 c.c. 3 per cent. suspension of ox or sheep red cells sensitised with five doses of immune body from the rabbit) usually occurs when these are added to a mixture of the amount of heated serum commonly employed (0.025 to 0.05 c.c. in 0.5 c.c. saline) along with 1½ to 2 doses of guinea-pig's complement previously incubated for 1½ hours at 37° C., the dose of complement being the amount which causes complete lysis when incubated with saline in the absence of the human serum. Scattered references to unusually anti-complementary sera occur in the literature, thus Thomsen and Bjarnhjedinsson recorded that the sera of lepers possessed very considerable anti-complementary action and this has been confirmed by Mathis and Beaujean among others. But excluding special treatment of sera, *e.g.* prolonged keeping as found by Browning and Mackenzie and confirmed by Zinsser and Johnson, or heating at higher temperatures, pronounced anti-complementary action is very rare.

In this paper we wish to draw attention to two instances of sera showing considerable anti-complementary power; in neither of these could any certain explanation of this abnormality be found either in the condition of the patient or in the mode of treatment of the serum. These are the only cases of the kind which we have observed in the performance of many thousands of Wassermann tests. The practical importance of this anti-complementary action is obvious, since such sera would inevitably be regarded as giving a positive Wassermann reaction if no control test of the serum alone were made.

The sera were inactivated as usual by heating for half-an-hour at 56° C. The serum control tubes contained 0.5 c.c. saline, 0.05 c.c. patient's serum, and amounts of complement as shown in the tables. The antigen employed in the Wassermann tests was an alcoholic extract of human heart plus cholesterol. The phenomenon was found to be independent of the species of red corpuscles employed, since the examination of Case I was carried out with those of the sheep, and that of Case II with those of the ox. The sera



were preserved after the first examination by freezing at from  $-10^{\circ}$  to  $-20^{\circ}$  C. All the results quoted below are based upon repeated examinations of which single examples only are given in the tables.

*Case I.* Five samples of this serum (A, B, C, D and E) were examined.

The patient contracted trench fever in June, 1918; on August 3rd of that year he received a gun-shot wound in the right forearm which caused considerable injury to the flexor muscles and tendons; the wound was dirty when the patient came under treatment; the damaged portions were then excised. A week later severe haemorrhage from the wound occurred and 1000 c.c. of blood of Group II was given by transfusion.

On November 14th excision of the scar and secondary suture were carried out; sample A was taken before the anaesthesia, and sample B twelve hours later; the temperature was at this time within normal limits. After the operation the arm became oedematous and showed lymphangitis, and five days later the patient had a febrile attack, the temperature rising to  $105^{\circ}$  F. Sample C was obtained on November 29th, when the temperature had been normal for a week. Sample D was taken on December 19th, and E at the beginning of March, 1919. The state of the serum was thus observed over a period of four months. The patient presented no clinical evidence of syphilis.

The estimations of the full anti-complementary power of the serum are shown in Table I, and the results of the Wassermann tests carried out in the ordinary way in Table II.

Table I. Case I. *Anti-complementary Action of Serum.*

Date when sample taken	Complement, No. of M.H.D. ...	1.5	3	4.5	6	7.5
16. xi. 18	Sample A ... lysis	none	trace	marked	very marked	almost complete
	Sample B ... „	none	trace	distinct	very marked	almost complete
29. xi. 18	Sample C ... „	none	faint trace	trace	very marked	just complete
Complement, No. of M.H.D. ...						
19. xii. 18	Sample D ... lysis	1.3	2.6	4	5.2	—
		none	trace	just complete	complete	—
Complement, No. of M.H.D. ...						
1. iii. 19	Sample E ... lysis	2	—	—	—	—
	Samples A, B and C mixed and kept frozen since Nov. 1918; re-examined 3. iii. 19	complete	—	—	—	—

(1) Table I shows that samples A, B and C (Nov. 16th and 29th) showed almost identical anti-complementary action, lysis being in each case not quite complete in the presence of 7.5 M.H.D. of complement; the anaesthetic administered between the withdrawal of samples A and B had therefore no influence upon the condition. All other sera tested with the same batch of complement showed, as is almost invariably the case, complete or almost complete lysis with 1.5 or 2 M.H.D.; the inhibitory action of the serum in question must therefore have been six or seven times greater than normal.



Sample D, taken three weeks after sample C, showed just complete lysis with 4 M.H.D.; the anti-complementary power had therefore undergone diminution, though it was still distinctly abnormal. In sample E, taken two months after D, the normal condition was found to be re-established, lysis being complete with 2 M.H.D. The observations show, therefore, the decline and disappearance of the inhibitory character of the serum in the course of four months. These results obtained by one of us were practically duplicated by the other working independently at another laboratory. One other point may be mentioned: after the experiments carried out in November, 1918, the residue of the heated samples A, B and C were mixed and kept frozen at  $-10^{\circ}$  to  $-20^{\circ}$  C. until the following March, and then tested in the same way as before: the inhibition of lysis was found to have disappeared completely (Table I). This property must therefore be due to some quite unstable factor, since freezing is very effectual in preserving many of the properties of serum.

(2) The results of the Wassermann tests carried out in the usual manner with these sera are given in Table II; a comparison of these with the data given in Table I shows that the amounts of complement fixed in the presence of antigen (Table II) are in each of the five tests practically identical with

Table II. Case I. *Wassermann Tests.*

				Serum control
Complement, No. of M.H.D.	1.5	3	4.5	1.5
Sample A ... lysis	none	trace	distinct	faint trace
Sample B ... „	none	trace	distinct	faint trace
Negative control serum „	almost complete	complete	—	complete
Antigen control ... „	distinct	complete	—	—
Complement, No. of M.H.D.	1.5	3	4.5	1.5
Sample C ... lysis	none	faint trace	trace	none
Negative control serum „	complete	—	—	complete
Antigen control ... „	almost complete	complete	—	—
Complement, No. of M.H.D.	1.5	3	4.5	1.5
Sample D ... lysis	faint trace	marked	complete	faint trace
Negative control serum „	complete	—	—	complete
Antigen control ... „	complete	—	—	—
Complement, No. of M.H.D.	2	4	—	2
Sample E ... lysis	complete	—	—	complete
Negative control serum „	just complete	complete	—	complete
Antigen control ... „	complete	—	—	—

those inhibited by the sera alone (Table I). For instance, sample C gave a trace of lysis with 4.5 M.H.D. of complement both in the presence and in the absence of antigen. The Wassermann reaction was, therefore, negative: as was mentioned above, the patient showed no clinical evidence of syphilis. The results with the negative control serum included in Table II show that sera such as samples A, B, C and D would inevitably be regarded as giving a positive Wassermann reaction if no control observations were made with

the serum alone; whereas the employment of proper serum controls, such as are recorded in the last column of the table, causes the abnormality to be at once detected.

The apparent positive Wassermann reaction diminished *pari passu* with the loss of anti-complementary power, until in the last sample (E) the behaviour of the serum is seen to be practically identical with that of the negative control. Incidentally the results in Table II show that the administration of the anaesthetic (Nov. 16, samples A and B) had no influence upon the strength of the Wassermann reaction. This is of interest in view of statements that an anaesthetic may cause the serum to react positively.

Case II. Female, aged 56. As regards the clinical history of the case, we have been able to learn no more than that the patient showed "mental symptoms." The serum (sample A, Table III) showed somewhat less inhibitory power than did the first samples in Case I, lysis being complete with 5 M.H.D.

Table III.

Case II. (1) *Anti-complementary Action of Serum.*

Date	Complement, No. of M.H.D.	2	3	5
3. iii. 19	Sample A ... lysis	none	distinct	complete
2. iv. 19	Sample B ... „	complete	—	—

(2) *Wassermann Test.*

Complement, No. of M.H.D.				Serum control
				1·5
3. iii. 19	Sample A ... lysis	none	none	faint trace
	Negative control serum „	complete	—	complete
	Antigen control „	complete	—	—
Complement, No. of M.H.D.				2
	Sample B ... lysis	none	none	complete
2. iv. 19	Negative control serum „	complete	—	complete
	Antigen control „	complete	—	—

of complement. In contrast to Case I, the Wassermann reaction was found to be positive, no lysis occurring in the presence of antigen with 4·5 M.H.D., whereas in the absence of antigen lysis would no doubt have been almost if not quite complete with this amount. All other sera tested on this occasion showed complete or practically complete lysis with 2 M.H.D. The serum was somewhat deeply tinted with haemoglobin, but we have examined hundreds of such sera from partially lysed bloods without encountering any other instances of anti-complementary action.

A month later a second sample (B), free from haemoglobin, was examined. In the meantime the patient had received anti-syphilitic treatment ("914" and calomel), an injection having been given a week before the withdrawal of the blood. The Wassermann reaction was found to be as strongly positive as before (no lysis with 6 M.H.D.), but the abnormal anti-complementary power had disappeared, lysis being complete in the serum control tube with 2 M.H.D.



The second case thus resembles the first in that the anti-complementary property was transient only, disappearing in the course of from one to three months. That this fixation of complement is independent of that which is the basis of the Wassermann reaction is shown by the facts that (1) the Wassermann was in Case I negative and in Case II positive, and (2) in Case II the anti-complementary power disappeared while the Wassermann remained positive. The two cases do not present any common feature which would suggest the cause of the abnormality in question.

#### SUMMARY.

Two instances are described of sera showing abnormal anti-complementary power. The amount of complement fixed by the serum was in the one case about six times, in the other about four times, greater than is normal. Examination of subsequent specimens from the patients showed that this inhibitory character was transient only. No feature common to the two cases was found to which the abnormality could be attributed. Attention is drawn to the rarity of this condition, but it is of practical importance in that such sera would be regarded as giving a positive Wassermann reaction if their behaviour in the absence of antigen were not observed.

#### REFERENCES.

- BROWNING and MACKENZIE (1908). *Journ. of Path. and Bact.* XIII. 325; see also *Recent Advances in the Diagnosis and Treatment of Syphilis*, London, 1912.  
MATHIS and BEAUJEAN (1915). *Bull. Soc. Pathol. exot.* VIII. 252.  
THOMSEN and BJARNHJEDINSSON (1910). *Zeitschr. f. Immunitätsforsch. Orig.* VII. 414.  
ZINSSER and JOHNSON (1911). *Journ. Exp. Med.* XIII. 31.



## OBSERVATIONS ON THE CULTIVATION OF TYPHOID AND PARATYPHOID BACILLI FROM THE STOOLS WITH SPECIAL REFERENCE TO THE BRILLIANT GREEN ENRICHMENT METHOD.

BY CAPTAIN J. W. McLEOD, R.A.M.C., 8th MOBILE LABORATORY.

THE question of standardising bacteriological methods is at present under discussion and, although it is most undesirable that any one method should be forced on all bacteriologists, it is certainly reasonable that any method which has given excellent results in the hands of a number of independent workers should have a strong recommendation.

Browning, Gilmour, and McKie (1913) described a method for isolating Typhoid bacilli from the faeces by means of an incubation in dilute solutions of Brilliant Green prior to plating, and the value of the method has been investigated in many laboratories in the course of this war. Glynn and his collaborators (1917) summarised the published work dealing with this subject which had appeared up to the date of their report, and they expressed the general conclusion that "the available evidence indicates that the advantage of Brilliant Green, certainly of Browning's simplified technique, is not sufficiently established to justify its being recommended as an additional routine method in laboratories where enterics are examined, especially having regard to the slight extra labour and cost." In as much as the method has been, in my experience, one of the most valuable modifications of bacteriological technique which have been introduced and since it would seem to me to be a misfortune if the opinion expressed in the report quoted should deter any who are unfamiliar with the method from giving it a trial, I have not hesitated to publish the results which follow, although the conclusions may seem to some to be already well established.

The observations relate to the work of a Mobile Laboratory during the last four years. The opportunities for observing cases of infection of the Typhoid group have been the following: (i) a series of paratyphoid infections in the troops withdrawn from the Ypres sector to the area north of Albert in the summer of 1915; (ii) a small epidemic of typhoid amongst the civilians east of Doullens at the same period; (iii) a small epidemic of paratyphoid infection amongst the troops of a division which returned from Egypt in the early part of 1916; (iv) very occasional cases of typhoid or paratyphoid infections occurring amongst the troops or civilians in the area before and behind Cassell in 1917 and 1918; (v) a considerable outbreak of typhoid

infections amongst German civilians at Euskirchen in the end of 1918 and the beginning of 1919.

The observations made in the first two groups were mostly limited to blood culture, but in a considerable proportion of cases in the other groups a careful comparison between the value of direct plating and of Brilliant Green enrichment was made. In 1916 one tube only of Brilliant Green was used, a 1/250,000 dilution, and the method compared was that described by Ledingham and Arkwright (1912) with the modification that only one, not two or three plates were used for each specimen. In 1917, 1918 and 1919 1/250,000 and 1/500,000 dilutions of the dye were employed and the comparison was made with a literally direct method, *i.e.* the plate was inoculated with a small portion of faeces and spread immediately. The results of all investigations up to the end of 1918 in which the direct and Brilliant Green methods were compared are set down in Table I. These observations refer chiefly to paratyphoid infections and it is seen that out of the 16 results obtained 15 were

Table I.

Date	Name	Result	
		Brilliant Green	Direct
30-4-16	Driver S.	Paratyphosus B, numerous colonies	Paratyphosus B, scanty colonies
30-4-16	Pte. C.	Paratyphosus B, numerous colonies	Paratyphosus B, scanty colonies
21-5-16	Pte. W.	Paratyphosus B, numerous colonies	Paratyphosus B, scanty colonies
30-5-16	Pte. Lambert (1)	Paratyphosus A, nearly pure culture	Negative
	„ (2)	Paratyphosus A, one colony	Negative
	„ (3)	Paratyphosus A, nearly pure culture	Negative
12-6-16	Pte. S.	Paratyphosus B, colonies	Paratyphosus B, colonies
5-7-16	Pte. A.	Paratyphosus B, numerous colonies	Paratyphosus B, scanty colonies
24-8-16	Pte. F.	(1) Paratyphosus B, colonies	Negative
		(2) Paratyphosus B, colonies	Negative
*15-7-17	Pte. S.	Negative	<i>B. typhosus</i>
26-7-17	Pte. S.	Paratyphosus B	Negative
16-9-17	Pte. D.	Paratyphosus B, about 100 colonies	Paratyphosus B, about 20 colonies
29-11-17	Rflm. D.	Paratyphosus B	Negative
18-7-18	Civilian	Paratyphosus B, isolated	Negative
29-9-18	Civilian	<i>B. typhosus</i>	Negative

\* A tube of Brilliant Green solution which had been standing for some time and become discoloured was used on this occasion.

positive by the Brilliant Green method and only 7 out of 16 were positive by the direct method.

It was the case of Lambert (Table I) in this group which brought out the value of the method in the most striking way. An investigation had been required of all possible carriers in this man's regiment on account of a rather severe outbreak of paratyphoid infection. After investigating without success the stools and urines of 60 other men, I obtained an almost pure culture of



Paratyphoid A from the Brilliant Green tube of this man's stool but nothing except *B. coli* and few colonies of coarse non-lactose fermenters from the direct plate. The examination was repeated twice with the results cited in Table I confirming the original finding.

The man's history was that he had had a sharp attack of diarrhoea in Egypt, and had suffered from irregularity of the bowels ever since. The regimental epidemic had been a mixed one, "A" and "B" infections occurring simultaneously, but the latter predominating. No more cases of "A" infection occurred after the detection of Lambert. A continued investigation for a "B" carrier was unsuccessful; the "B" infections gradually died out, however, and were possibly all due to case to case contact.

In view of the above result I find it impossible to accept the opinion expressed by Glynn and his collaborators, as quoted above. Extra trouble and cost would have been involved by omitting the use of the Brilliant Green method in this case, and at least one month's fruitless work would have had to be carried out and possibly a continued series of paratyphoid "A" infections would have occurred in the regiment.

The claim, however, originally made by Browning, Gilmour and McKie for the Brilliant Green method was not only that it facilitated the isolation of paratyphoid bacilli but that of typhoid bacilli. Most of those who from their practical experience of the method in this war have written in its favour, only recommend it, however, in respect of the isolation of the paratyphoid bacilli (Stokes and Clark, 1916; Fletcher, 1917).

Table II.

Name	Stool		Urine	
	Direct	Brilliant Green	Direct	Brilliant Green
Sophie U. ...	Abundant Paratyph. B	Abundant Paratyph. B	Abundant Paratyph. B	Abundant Paratyph. B
Gertde. M. ...	Negative	Scanty colonies <i>B. typhosus</i>	Negative	Negative
Christ. D. ...	Approx. 10 col. of <i>B. coli</i> to 1 of <i>B. typhosus</i>	Approx. 1 col. of <i>B. coli</i> to 4 of <i>B. typhosus</i>	Negative	Negative
Schwester A. ...	One or two col. <i>B. typhosus</i> , <i>B. coli</i> abundant	Approx. 5 col. <i>B. typhosus</i> to 1 col. <i>B. coli</i>	Negative	A few col. <i>B. typhosus</i>
Frau S. ...	Negative	A few col. <i>B. typhosus</i>	Negative	Negative
Jacob S. ...	Negative	Paratyph. B	Negative	Negative
Heinrich C. ...	Negative	<i>B. typhosus</i>	Negative	Negative
Helene P. ...	<i>B. typhosus</i>	Negative	—	—
Frau S. (2nd exp.) ...	Negative	<i>B. typhosus</i>	—	—
Frau K. ...	Negative	<i>B. typhosus</i>	—	—
Frau U. ...	<i>B. typhosus</i>	<i>B. typhosus</i>	—	—
Aug. U. ...	<i>B. typhosus</i>	<i>B. typhosus</i>	—	—
Frau K. (2nd exp.) ...	Negative	<i>B. typhosus</i>	—	—
Baxter, 1st spec. ...	Negative	<i>B. typhosus</i>	—	—
„ 2nd spec. ...	<i>B. typhosus</i> col. scanty	<i>B. typhosus</i> col. numerous	—	—



The occurrence of the civilian epidemic already mentioned in the early part of 1919 at Euskirchen, a small town in the British area of occupation west of the Rhine, afforded a good opportunity of retesting the value of the Brilliant Green enrichment method in cases of infection with *B. typhosus*.

The results obtained are given in Table II.

Excluding the two paratyphoid infections, there were 14 specimens derived from 11 different cases, 13 of stool and one of urine, in which a positive result was obtained. In 13 of these the result was positive by the Brilliant Green method, whereas in 6 only by the direct method. In my experience therefore the original claim for the efficacy of the method in isolating *B. typhosus* from the stool is fully vindicated.

#### *Technique.*

A few words about the technique appear relevant since it is probably the source of varying results amongst different workers. The dilutions of Brilliant Green in peptone water have been made by adding with a sterile graduated pipette the requisite quantities of  $\frac{1}{5000}$  solution of Brilliant Green to sterile tubes of peptone water each containing 10 c.c. Solutions of Brilliant Green in peptone water were not autoclaved although I have no proof that this has any deleterious effect. The original technique was persisted in as it had given good results. The Brilliant Green tubes were always inoculated copiously, much more material being transplanted than could be adequately spread directly on several plates.

The peptone water used was capable of yielding a rapid and copious growth of *B. typhosus*, and suitable Brilliant Green was employed. These two factors are important. If peptone water is neutralised by a fixed addition of alkali and not titrated, it may easily happen that in frequent moving a laboratory will strike some water supply of an unusual grade of alkalinity or acidity, which if used to prepare peptone water according to formula will yield a product incapable of promoting a rapid growth of *B. typhosus*.

A number of bottles of "Brilliant Green" crystals have been issued through the Army Depots of Medical Supplies which have neither had the crystalline appearance nor the antiseptic properties of Brilliant Green. An old bottle of Gröbler's preparation has been used throughout in these investigations, and it does not seem to me that any criticism of this method is pertinent unless the work is carried out with Gröbler's Brilliant Green or with a specimen which has been proved equal to it in parallel experiment.

#### REFERENCES.

- BROWNING, GILMOUR and McKIE (1913). *Journ. of Hygiene*, XIII. p. 335.  
FLETCHER (1917). *Journal Roy. Army Med. Corps*, XXIX. pp. 544-549.  
GLYNN, BERRIDGE, FOLEY, PRICE and ROBINSON (1917). *Report to Medical Research Committee*, No. 7, p. 5.  
LEDINGHAM and ARKWRIGHT (1912). *The Carrier Problem in Infectious Disease*, p. 104.  
STOKES and CLARK (1916). *Journal Roy. Army Med. Corps*, XXVI. pp. 466-467.

THE BACTERIAL CONTENT OF THE AIR IN ARMY  
SLEEPING HUTS, WITH ESPECIAL REFERENCE TO  
THE MENINGOCOCCUS.

BY A. J. EAGLETON, M.D.

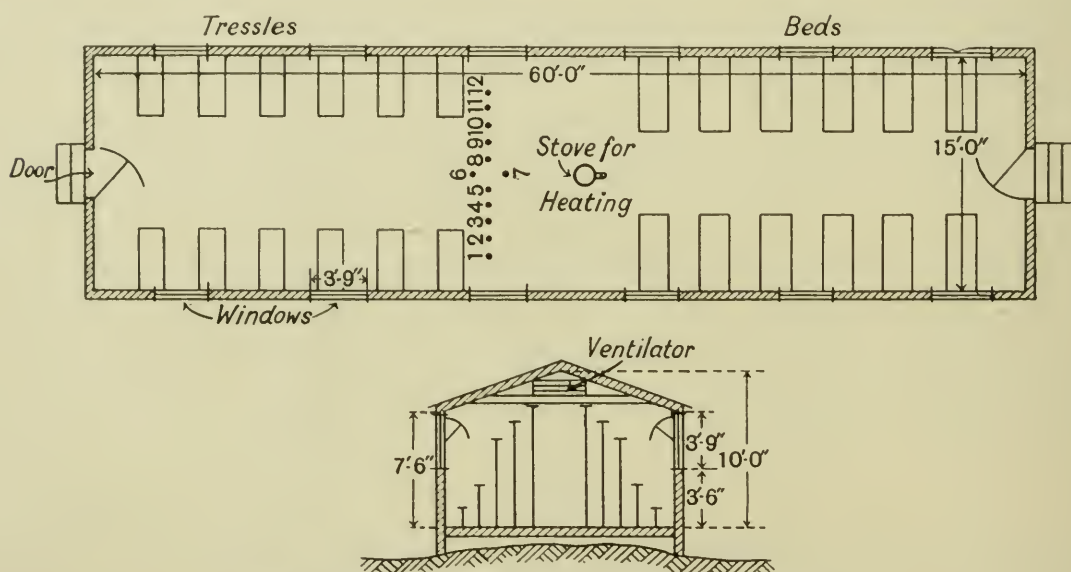
(Formerly Officer i/c Laboratory, Wylye Area.)

(With 1 Diagram.)

THE following experiments were carried out in October and November, 1917. They have not been published hitherto as it was hoped that they might be extended and revised.

The onset of the influenza epidemic, the armistice and the resulting dispersal of troops prevented this, and in so far as some of the conclusions arrived at seem of no little importance, it has been thought advisable to publish the results of the work done, incomplete though it is.

The first series of experiments I–VIII deals with the number of organisms found on plates exposed during the night in men’s sleeping huts, Barrack Design 230/14. The same hut was always used, and the number of men in the hut was always 22.



### Men's Sleeping Hut: Barrack Design 230/14.

This diagram shows the ground plan and vertical section of the type of hut in which the experiments were conducted.

The "beds" used were either ward beds or plank trestles, but no appreciable difference in the bacterial content of the hut could be detected whichever type was employed. It will be noticed that there was a ventilator in the roof extending the entire length of the hut and that there were six windows on each side, the upper half of which could be opened. There was no ground ventilation but the huts were floored with planks and raised above the grass on wooden supports.

*Experiment I.*

Plates exposed 10.30 p.m., 25.10.17.

Plates removed to incubator 6.0 a.m., 26.10.17.

Temperature in hut: Maximum 58° F., Minimum 48° F.

Plates	Level	Colonies
1, 12	1 foot above ground	All plates showed innumerable colonies; no mould or other gross contamination.
2, 11	3    "    "	
3, 10	5    "    "	
4, 9	6    "    "	
5, 8	7    "    "	
6, 7	Ground level	

*Experiment II.*

Plates exposed 10.30 p.m., 28.10.17.

Plates removed 6.0 a.m., 29.10.17.

Temperature in hut: Maximum 56° F., Minimum 44° F.

Plates	Level	Colonies
1, 2	8 feet above floor	All plates showed innumerable colonies; Nos. 5 and 9 were overgrown with mould.
3, 4	7.5 "    "    (inverted)	
5	7    "    "	
6	6    "    "	
7	5    "    "	
8	4    "    "	
9	3    "    "	
10	2    "    "	
11	1    "    "	
12	Floor level	

*Experiment III.*

A comparison of the effect of beds and trestles. The height of the men's heads above the floor was 2 feet 3 inches and 1 foot 3 inches in the two groups.

Plates	Level	Colonies
2, 6, 12, 14	Ground level	All plates in both sets showed innumerable colonies; none overgrown.
4	2 feet	
5	1½ "    "	
1	2½ "    "	
13	3    "    "	
9	3½ "    "	
11	4    "    "	
7	4½ "    "	
8	5    "    "	
15	6    "    "	

Two sets of plates used, one between beds and the other between trestles.



*Meningococcus**Experiment IV.*

An exact repetition of Exp. III with the same result.

*Experiments V and VI.*

Two identical experiments to show the number of bacteria in the air in spaces between beds and trestles at higher levels.

Plates exposed 19.10.17, midnight.

Plates removed 20.10.17, 6.0 a.m.

Temperature: Maximum 58° F., Minimum 40° F.

Plates	Level	Colonies
5	7 feet	In both sets innumerable colonies; none overgrown.
4	6½ „	
1	6 „	
6	5 „	
3	4 „	
2	3 „	

Two sets as in Experiments III and IV.

*Experiment VII.*

Plates placed as high as possible in intervals between beds.

Plates exposed 10.30 p.m., 20.10.17.

Plates removed 6.0 a.m., 21.10.17.

Temperature: Maximum 50° F. (?), Minimum 49° F.

Plates	Level	Colonies
1, 2, 3, 4	8¼ feet	All plates showed innumerable colonies, except 5 and 7 (both inverted).
7, 8, inverted	7¼ „	
9, 10	7 „	
6, 5, inverted	6¼ „	
		5, 250 colonies
		7, 360 „

*Experiment VIII.*

Same as VII, only trestles instead of beds.

Plates exposed 21.10.17, midnight.

Plates removed 6.0 a.m., 22.10.17.

Temperature: Maximum 56° F., Minimum 48° F.

All plates showed innumerable colonies.

Experiments I–VIII show therefore that in the type of army hut investigated the bacterial content of the air during sleeping hours is very high and that the air throughout the hut is uniformly infected.

*Experiment IX.*

A larger type of hut used. Plates placed all on ground level.

Plates exposed 10.10.17, 10.0 p.m.

Plates removed 11.10.17, 6.0 a.m.

Temperature: Maximum 55° F., Minimum 45° F.

Plates	Position	Colonies
2, 3, 5, 6	Between trestles	Uncountable
1	In centre of room	48
4	" "	30

### *Experiment X.*

Hospital ward. Space between beds 2 feet. Plates placed between beds.

Plates exposed 14.10.17, 9.30 p.m.

Plates removed 15.10.17, 4.30 a.m.

Temperature: Maximum 54° F., Minimum 43° F.

Plates	Level	Colonies
1	Ground	152
2	"	164
3	"	Overgrown
4	"	"
5	1 foot	176
6	1 "	Overgrown
7	1½ feet	184
8	1½ "	Overgrown
9	2 "	166
10	2 "	Overgrown
11	2½ "	"
12	2½ "	127
13	3 "	101

It is seen that, although the bacterial content of the air is less than in the ordinary army hut, still the number of colonies obtained is very considerable both at ground level and at 3 feet.

### *Experiment XI.*

Comparison of condition in a half hut, type 230/14, when 5 feet was allowed between each bed, the men being carriers of meningococcus.

Plates exposed 13.10.17, 10.0 p.m.

Plates removed 14.10.17, 6.30 a.m.

Temperature: Maximum 38° F., Minimum 36° F.

Plates	Level	Colonies
10	Ground	540
11	"	496
12	"	450
13	"	450
1	1 foot	Overgrown
2	1 "	546
3	1½ feet	304
4	1½ "	? slightly overgrown
5	2 "	308
6	2 "	300
7	2½ "	300
8	2½ "	276
9	3 "	250

Meningococci were present in No. 4. In all cases where meningococci are stated to be present, they were proved by agglutination to be genuine strains.

*Meningococcus**Experiment XII.*

To show the presence of meningococci in the air. Patients were all known carriers.

Plates exposed 11.10.17, 10.0 p.m.

Plates removed 12.10.17, 6.30 a.m.

Temperature: Maximum 53° F., Minimum 47° F.

*Plates.* Six exposed on ground level, in intervals between beds. All showed innumerable colonies. Meningococci present, but only one plate showed several colonies.

*Experiment XIII.*

More exact experiment on conveyance of meningococcus. Chronic carrier lying on trestle for 5 minutes, coughing periodically. Plates arranged on the ground at different distances from his mouth, which was 1 foot 9 inches above the floor. The room had been previously sprayed with formalin and the floor scrubbed and cresoled.

Plates	Distance from subject in feet	Meningococci
1, 2	6	overgrown
3, 4	7	„
5, 6	8	„
7, 8	9	„
9, 10	10	negative
11, 12	11	„
13, 14	12	overgrown
15, 16	13	positive
17, 18	14	overgrown

*Experiment XIV.*

Chronic carrier sleeping with face towards plates. Plates all on ground level. Same preparation of room as in Experiment XIII.

Plates exposed 12.10.17, 10.0 p.m.

Plates removed 13.10.17, 6.30 a.m.

Plates	Distance in feet	Meningococci	Colonies
1	3	? not proven	Uncountable
2	3	+	320
3	5	+	276
4	5	+	360
5	6	overgrown	
6	6	„	
7	7	„	
8	7	—	372
9	8	—	288
10	8	—	204
11	9	—	172
12	9	—	190
13	10	overgrown	
14	10	„	
15	11	—	160
16	11	—	140
17	12	overgrown	
18	12	—	146



*Experiment XV.*

Exact repetition of Experiment XIV.

Plates exposed 9.10.17, 10.0 p.m.

Plates removed 10.10.17, 6.30 a.m.

Plates 1, 3 and 4 showed meningococci, i.e. at distances of 3 and 5 feet.

*Experiment XVI.*

Same as preceding. Plates at different distances and levels from face of carrier.

Plates exposed 15.10.17, 10.0 p.m.

Plates removed 16.10.17, 6.30 a.m.

Temperature: Maximum 53° F., Minimum 50° F.

	Plates	Level	Colonies	Meningococci
	7 (1 foot from subject)	2½ feet	45	+
20	„	4 „	40	-
13	„	5 „	overgrown	
3	„	1½ „	„	
11	„	3½ „	44	+
	6 (2 feet from subject)	6 „	overgrown	
2	„	1 „	30	-
15	„	5½ „	38	+
8	„	3 „	45	+
	4 (3 feet from subject)	5 „	overgrown	
18	„	3½ „	54	+
14	„	3 „	overgrown	
12	„	5½ „	„	
17	„	1½ „	smashed	
	16 (4 feet from subject)	2½ „	overgrown	
5	„	4½ „	„	
1	„	2 „	150	+
	19 (5 feet from subject)	4 „	60	-
9	„	4½ „	overgrown	
21	„	2 „	56	-
22	„	1 „	56	-
10	„	6 „	overgrown	

## SUMMARY.

*Experiments I-VIII.*

I shows that under the conditions shown the plates were covered with innumerable colonies 7 feet above ground level to ground level. The beds and trestles were used to keep the hut always uniform.

II shows that 8 feet above ground, i.e. on top of the crossbeams, the same conditions obtain.

III, IV, V, and VI show that the same condition exists in the area between the beds or trestles. The beds make no difference, better or worse.

VII shows practically the same condition high up on the beam between the beds. The inverted plates naturally show less colonies, as the organisms mostly fall on to the plates.

VIII confirms the above.

There is only one conclusion to draw from these experiments, and that is that practically speaking there is no difference in the number of organisms on the ground floor and 8 feet above it, in the ordinary men's sleeping huts, Barrack Design 230/14.

*Experiments IX-XII* are shown for comparative reasons.

IX shows a better condition in a broader hut, although more men were sleeping in it.

X shows the superiority of the ward buildings. The beds were only 2 feet apart and chosen specially to show effect of overcrowding.

XI shows effect of bed space and low temperature. It also shows possibility of transfer of meningococci. The meningococci were proved by agglutination tests.

XII was a control on XI. The effect of the higher temperature is seen, also possibility of spread of meningococci.

#### *Experiments XIII-XVI.*

These are more exact experiments on distance to which meningococci can be carried. "Spraying capacity of patient."

XIII. By coughing it was carried 13 feet. Several like experiments were done, but owing to overgrowth by *subtilis*, etc., this was the only one where the organism could be proved by agglutination. Others showed suggestive colonies but they could not be isolated.

XIV. This showed that in ordinary sleep a carrier can spray to a distance of 5 feet.

XV confirms above, and from these two and several other negative experiments it seems justifiable to conclude that the "spraying capacity" of a carrier during ordinary sleep is 5 feet along the level or just below it.

XVI shows that meningococci are carried during sleep:

1 foot at a height of 2 ft 6 in.			
1	„	„	3 „ 6 „
2	„	„	5 „ 6 „
2	„	„	3 „ —
3	„	„	3 „ 6 „
4	„	„	2 „ —
5	„	nothing above ground level	

so that we may say that in ordinary sleep the organism is not carried more than 5 feet, but in violent coughing it may be carried three times this distance.

The conclusions to be drawn from the above experiments, limited as they are, seem to be the following:

(1) In the ordinary infantry sleeping hut, there is a stagnant well of infected air in which the men sleep.

(2) This dead space extends up to 8 feet above the ground level.

(3) The wider the hut and the lower the temperature, the better the condition of the air.

(4) More adequate ventilation is needed; probably this could be obtained by ground ventilation.

(5) The meningococcus can be carried at night from a carrier to his neighbours unless the bed space is more than 5 feet.

(6) The spraying capacity of a carrier varies between 5 and 15 feet, but is 5 feet during ordinary sleep.

(7) The meningococcus is carried in the spray to a much shorter distance than many other organisms.

In conclusion, I have the pleasant task of thanking those who have helped me to carry out this piece of work. Colonel Morse, R.A.M.C., arranged for the use of the hut and the discipline of the subjects. Capt. Welsford, Senior Sanitary Officer, interested himself in the work and obtained for me the services of Lt. Hasnip who provided the diagrams of the huts. Finally, Colonel Mervyn Gordon criticised the earlier experiments and supplied suggestions for new work, which unfortunately I was unable to carry out.



AN EXPERIMENTAL INVESTIGATION OF AN  
AUSTRALIAN EPIDEMIC OF ACUTE  
ENCEPHALO-MYELITIS<sup>1</sup>.

BY J. BURTON CLELAND, M.D., AND A. W. CAMPBELL, M.D.

(From the Microbiological Laboratory, Department of Public Health,  
New South Wales.)

CONTENTS

	PAGE
I. Introduction . . . . .	274
II. Inoculation, Materials and Methods . . . . .	274
III. Details as to the Viruses employed . . . . .	276
(1) Estimated Day of Illness when the Virus was obtained .	276
(2) Hours after Death when the Virus was Removed from the Body . . . . .	276
(3) Period during which the Virus was Preserved <i>in vitro</i> .	276
(4) Menstruum in which the Virus was Preserved . . . .	276
IV. The Disease as manifested in Monkeys ( <i>Macacus rhesus</i> ) . . .	276
(1) The Incubation Period . . . . .	276
(2) Length of Illness . . . . .	277
(3) The General Course of the Disease . . . . .	277
(4) The Symptoms and Signs manifested on the First Day of Illness . . . . .	277
(5) Later Manifestations . . . . .	277
V. The Disease as manifested in Sheep . . . . .	278
(1) Incubation Period and General Course . . . . .	278
(2) Analysis of Symptoms and Signs . . . . .	279
(3) Sheep Surviving Intracerebral Inoculation but showing slight symptoms, probably of Encephalo-myelitis .	280
(4) Inoculation of Sheep in Series.—Insusceptibility of some Animals . . . . .	282
(5) Sheep Showing Natural Immunity to Intracerebral Inocu- lation of the Virus . . . . .	284
VI. The Disease as manifested in the Calf . . . . .	285
VII. The Disease as manifested in the Horse . . . . .	285
VIII. Other Experiments and Various Inferences Drawn . . . .	285
(1) The Treatment of the Virus with various Sera before Intra- cerebral Inoculation into Monkeys . . . . .	285
(2) The Treatment of the Virus with various Sera before Intra- cerebral Inoculation into Sheep . . . . .	288

<sup>1</sup> A full epidemiological, clinical and histological account of the disease may be read in the Annual Report of the Microbiological Laboratory of the Department of Public Health, Sydney, N.S.W., for the year 1917.

	PAGE
(3) Experiments suggesting that the Virus is no longer present on the eighth or tenth day of Illness in Monkeys . . . . .	289
(4) Experiments showing presumed Dying out of the Virus during prolonged Storage in Glycerine in the Cold . . . . .	289
(5) Experiments possibly showing a Waning in Virulence of the Virus . . . . .	289
(6) Experiments suggesting the Production of Artificial Immunity . . . . .	290
IX. Summary and Conclusions . . . . .	290
X. Appendices . . . . .	291
I. Tabulation of the Kinds of Inoculations and Results in the respective Species of Animals . . . . .	291
II. Table of Monkey Inoculations. Positive Results . . . . .	294
III. Details of the Successful Inoculations in Individual Monkeys ( <i>Macacus rhesus</i> ) . . . . .	295
IV. Death of a Monkey in Twelve Days from Pathogenic Infection without Co-existent Evidence of Encephalo-mylitis . . . . .	298
V. Failure in Monkeys of Certain Intracerebral Inoculations of Human Material . . . . .	298
VI. Failure in Monkeys of Certain Intracerebral Inoculations of Brain and Spinal Cord from Monkeys . . . . .	300
VII. Failure of Intraperitoneal Inoculations . . . . .	301
VIII. Failure of Intrasciatic Inoculations . . . . .	301
IX. Failure of Pasteur-Chamberland F. Filtrates . . . . .	301
X. Failure of Cerebro-spinal Fluid to cause Infection . . . . .	302
XI. Failure of a Pasteur-Chamberland F. Filtrate of Fæces . . . . .	302
XII. Failure of a "Noguchi Culture" . . . . .	302
XIII. Failure in Monkeys of Intracerebral Inoculations from the Nasopharyngeal Swabs of Contacts and of a Case . . . . .	302
XIV. Failure in a Monkey of the Intracerebral Inoculation of Brain and Spinal Cord from a Horse . . . . .	303
XV. Failure of Intracerebral Inoculation of an Emulsion of Fowl Ticks ( <i>Argas persicus</i> ) . . . . .	303
XVI. Failure in a Monkey and a Sheep after the Introduction of Horse Serum into the Spinal Canal and Introduction of the Virus into a Vein . . . . .	303
XVII. Table of Sheep, Calf and Horse Inoculations. Positive Results with Death . . . . .	303
XVIII. Summary of Successful Inoculations in Sheep, a Calf and a Horse . . . . .	304
XIX. Sheep showing no Symptoms after an Intracerebral Inoculation of Brain and Spinal Cord from Human Cases . . . . .	306
XX. Sheep showing no Symptoms after the Intracerebral Inoculation of Brain and Spinal Cord from Infected Monkeys . . . . .	306
XXI. Sheep showing no Symptoms after the Intracerebral Inoculation of Brain and Spinal Cord from Infected Sheep . . . . .	307
XXII. Failure in a Sheep of an Intracerebral Inoculation of Dried Brain and Spinal Cord from a Positive Monkey . . . . .	307
XXIII. Failure in Sheep of a Berkefeld Filtrate from a Positive Monkey . . . . .	307
XXIV. Failure to Convey the Disease to a Sheep by Intranasal Swabbing . . . . .	307

	PAGE
XXV. Sheep Reinoculations . . . . .	307
(a) Successful Reinoculations . . . . .	308
(b) Unsuccessful Reinoculations . . . . .	309
XXVI. (a) Calves showing Symptoms of Illness, possibly due to Encephalo- myelitis, after Intracerebral Inoculation of Material from the Brain and Spinal Cord of a Positive Monkey or a Positive Horse	310
(b) Calf showing no Symptoms after Intracerebral Inoculation (a second inoculation) of Brain and Spinal Cord from a Positive Sheep . . . . .	311
XXVII. Failure to Convey the Disease to Dogs . . . . .	311
XXVIII. Failure to Convey the Disease to a Kitten by Intracerebral Inoculation	311
XXIX. Failure to Convey the Disease to Rabbits . . . . .	311
XXX. Doubtful Results in Guinea-pig Inoculations . . . . .	311
XXXI. Failure to Convey the Disease to a Hen by Intracerebral Inoculation	312
XXXII. Table showing the Various Animal Inoculations . . . . .	312

I. INTRODUCTION.

THIS investigation concerns an epidemic disease, which for convenience we have called “X disease,” prevalent in certain parts of New South Wales during the late summer of the years 1917 and 1918.

The disease was at first thought to be “acute poliomyelitis”—the Heine-Medin disease. Increased observation, however, revealed important discrepancies; thus (1) it was confined to “outback” towns or districts remote from one another and from the metropolis (Sydney), and did not synchronise with any metropolitan epidemic of “acute poliomyelitis”; (2) it was extremely fatal (we have notes of 134 cases, of which no less than 94 died); (3) it attacked a number of adults (34 cases); (4) signs of intense cerebro-spinal irritation (convulsions, rigidity, increased reflex activity, mental confusion and pyrexia) dominated the clinical picture, paralysis, intercurrent or residual, being infrequent; and (5) the histological picture differed from that of “acute poliomyelitis,” inasmuch as the changes were distributed throughout the central nervous system and did not fall with special intensity on the spinal cord.

Impressed by the peculiar features of the disease, we set in train the series of experiments herein recorded. The list comprises 62 experiments on monkeys, 52 on sheep, four on calves, one on a horse, and sundry experiments on dogs, kittens, rabbits, guinea-pigs and a fowl. Hence the research has been extensive. We may also say now that, while various side issues have been followed up with interesting results, the finding of chief importance is that the disease is communicable not only to the monkey, but to the sheep, the horse and the calf, that is, to animals which, so far as we are aware, have hitherto proved resistant to “acute poliomyelitis”—which is an additional discrepancy.

II. INOCULATION, MATERIALS AND METHODS.

The usual procedure in obtaining and maintaining the virus for inoculation purposes was the following:

At the autopsy of the human being or animal dead from the disease, thin



slices of tissue were taken from the frontal, parietal, occipital and temporo-sphenoidal regions of the cerebrum, from the cerebellum, from the pons and medulla, and from the cervical, dorsal and lumbar areas of the spinal cord. These were then straightway put into 33 per cent. glycerine diluted either with water, or in the earlier cases with normal saline solution, in which they were kept in an ice chest until wanted; or the material was at once emulsified with sterile powdered quartz in a mortar with the diluent, and stored similarly after a proper milky emulsion had been made. To preserve material from one or two of the earlier human cases the strength of the glycerine solution was 50 per cent. In some cases the emulsion was made with normal saline solution only when it was proposed immediately to inoculate a test animal. When small blocks of tissue had been preserved in glycerine, they were emulsified in a mortar just before inoculation. Pasteur-Chamberland F. and Berkefeld filtrates of this primary emulsion of the brain or spinal cord were sometimes used, whilst in other cases, after light centrifuging, the supernatant fluid was treated with various sera.

Other materials used for inoculation comprised swabbings from the nasopharynx emulsified in 33 per cent. glycerine solution, a Pasteur-Chamberland F. filtrate of faeces from a human case, and an emulsion of fowl ticks.

Nearly all the inoculations were made intracerebrally. The usual procedure was to tie the animal out on a frame and to anaesthetise it, first with chloroform and then with ether; in some cases chloroform alone was used. After cleaning the scalp and removing the hair, a small lineal incision was made in the parietal area down to the bone. Then by means of a small trephine, a puncture was made through the skull by means of the pin in the centre of the trephine, the teeth of the trephine keeping the pin in position. It was found better to use a trephine than an awl or other instrument. As a rule the time when the pin of the trephine had pierced the cranial vault could be easily estimated by the teeth of the trephine beginning to give. Occasionally, when a little undue force was used, the disc of bone loosened by the trephine became somewhat depressed. In one or two of the earlier cases the trephine tablet was removed. This was apt to lead, when the disease developed, to a hernia cerebri. The puncture having been made and by means of a surgical needle proved to be through the bone, a moderate sized needle of a large 5 c.c. syringe was inserted deeply into the cerebral substance, and about 1 c.c. of the emulsion injected. At one time the injection was made with some force, and the needle end was moved in various directions, with the deliberate object of destroying a certain amount of brain tissue in the neighbourhood of the injection and so facilitating the "taking" of the virus. It frequently happened that on withdrawing the syringe fluid escaped along the needle track but in several cases wherein this occurred the animals nevertheless contracted the disease.

The animals appeared to recover perfectly from the operation, usually within a few hours, and next day, with few exceptions, showed no results of

the operative treatment, in spite of the fact that definite injury must have been done to the brain.

In some of the earlier cases not only was the emulsion injected into the brain substance, but a certain amount was injected subdurally as well.

Intraperitoneal and intrasciatic inoculations were made in the usual way. In two cases lumbar puncture was performed, once in a monkey and once in a sheep, and a few c.c. of normal serum were injected into the spinal canal. Then an incision was made in the thigh or neck of the animal and about 0.5 c.c. of the emulsion was injected intravenously. No results followed from any of these inoculations. In the case of the intravenous introductions, the failure was possibly due to the small amount of virus employed.

### III. DETAILS AS TO THE VIRUSES EMPLOYED.

1. *Estimated day of illness when the virus was obtained.* This varied from one to eight days; in most cases it was obtained on the third to the fifth day. In the three human cases, the patient had died on the third, second and fifth days of the disease respectively.

2. *Hours after death when the virus was removed from the body.* With the exception of the cases in which the virus was obtained from human beings, the post-mortem examinations were made immediately after death or within a few hours. The three human cases had been dead 5, 12 and 21 hours respectively.

3. *Period during which the virus was preserved in vitro.* In a number of the animal experiments this was only a few hours. The longest periods during which it was preserved in glycerine and retained its activity were seven days in one human case and fifteen days in a monkey case.

4. *Menstruum in which the virus was preserved.* When the virus was immediately injected into a further test animal, it was usually suspended in normal saline solution. In most of the other cases it was preserved in 33 per cent. glycerine solution, the dilution of the glycerine being sometimes with normal saline solution, sometimes with sterile water. The percentage of glycerine was usually 33, but in one instance it was 50.

### IV. THE DISEASE AS MANIFESTED IN MONKEYS (*MACACUS RHESUS*).

Twenty monkeys altogether were successfully inoculated with the virus as proved by full histological examination after death. Details of the individual animals will be found in the Appendices.

1. *The incubation period.* This varied from 5 to 23 days, the average of the 20 cases being 10.25 days, or eliminating the very long incubation period of 23 days, 9.5 days. In only one instance was the incubation period as long as 23 days; in one it was 17 days; in one, 15 days; in two, 12 days; in three, 11 days; in three, 10 days; in two, 9 days; in one, 8 days; in three, 7 days; in one, 6 days; and in two, 5 days.



2. *Length of illness.* This varied from  $2\frac{1}{2}$  to 11 days. In most instances the animals were killed *in extremis*. In several instances it is possible, more especially in the case of A. 110, Monkey 3925, that the animal might have survived a considerably longer period, or have recovered. In the majority of cases, however, the animal had reached an almost moribund state in from 3 to 6 days.

3. *The general course of the disease.* In half, at least, of the affected monkeys, the first sign of the onset of the disease was a peculiar anxious facial expression. This sign might precede by a day other manifestations, or might be accompanied by evidences of incoördination and by exaggerated or irregular muscular movements and increased reflex action. In most instances there was a gradual progression of this incoördination and exaggerated muscular movement, sometimes accompanied by convulsive muscular contortions, and occasionally by true convulsions, until eventually the animal lay prostrate on the bottom of the cage. In some instances paresis of a limb, accompanied by incoördination, was a recognisable and early manifestation, and in one case prostration was the first indication of a "take." In some cases consciousness was apparently not lost until just before the animal was killed or died. The dominant features of the disease were exaggerated muscular movements and intense incoördination, though a few individual monkeys were quieter, and showed less incoördination, and more paresis.

4. *The symptoms and signs manifested on the first day of illness.* In ten of the twenty monkeys an anxious or frightened expression was easily recognisable on the first day of illness, and as already indicated was frequently the first intimation that the virus had "taken." In eight cases the monkeys were described as being nervous or jumpy, or as walking gingerly; in eight also there was definite incoördination of muscular movement. These two classes comprised fourteen of the twenty monkeys. Some spasticity of the limbs was noticed in one monkey, and convulsions were recorded in one. In six monkeys there was definite paresis of a limb, and in one the animal was found prostrate.

Regrouping the above signs, it will be found that on the first day of illness fifteen of the twenty monkeys showed exaggerated movements, incoördination, spasticity, or convulsions, whilst only seven showed paresis and none paralysis.

5. *Later manifestations.* The most note-worthy feature of these cases, seen in nineteen of the twenty monkeys, was an incoördination of movement of a form apparently due more to exaggeration of muscular efforts in attempts to balance than to paresis. In twelve of the animals, including eleven of the above, there were greatly exaggerated movements of limbs, and in three, including two of these twelve and one of the nineteen, definite convulsions. Thus, nineteen monkeys showed incoördination and thirteen exaggerated movements or convulsions, the whole twenty animals being included in these two categories.

The incoördination presented itself as inability to maintain the balance easily, and difficulty in quickly adjusting the limbs and hands to the various movements attempted. The exaggerated muscular movements were especially



noticeable when the animal attempted to jump, and became much more pronounced when it was disturbed and excited. Thus, in jumping on to a perch in the cage, the animal might miss the perch and pass below, or pass above and hit its head against the roof of the cage, or lose its hold and tumble off. In other cases, especially when disturbed, the animal would career wildly round the cage, dash its head against the sides, and apparently be unable to control the propulsive movements which it had initiated. Frequent bruising and injury, especially of the head, occurred under these circumstances. Often these movements were almost convulsive in character.

Definite convulsions occurred in three monkeys, and were merely a supreme exaggeration of the muscular movements already described. They were unaccompanied by a definite loss of consciousness. The animal during the attack would lie partly prostrate, or seized by violent contortions would rotate on the bottom of the cage, or possibly attempt to steady itself by efforts at grasping the bars. Such attacks sometimes lasted several minutes, and left the animal exhausted.

Irregular muscular contractions and twitches, apart from more purposive movements of limbs, were noticed in two animals. A general tremor, or a tremor of limbs or of the head, occurred in two cases, the movements in one resembling those of paralysis agitans. Some spasticity or rigidity of the limbs occurred in three and head retraction in five cases. Five of the animals uttered staccato or barking cries.

Paresis or paralysis was noted in fourteen of the twenty monkeys. In three there was slight paresis of a limb, in five there was marked paresis, and in six there appeared to be paralysis of a limb or other part. Two animals showed squint. In three there was ptosis, marked in two cases. The eyesight may possibly have been affected in one monkey. Three monkeys were drowsy, somnolent, or intensely sleepy.

Terminal prostration was manifested in nearly all the animals, varying from a slight degree, wherein there was inability to rise, with some remaining power of movement in a limb or limbs, to a condition so complete that perhaps an intelligent look in the eyes and slight movements of the tail tip or of a hand or foot were the only recognisable indications of life.

The temperature was frequently subnormal towards the end, sometimes markedly so, and in one case fell to 29° C.

#### V. THE DISEASE AS MANIFESTED IN SHEEP.

1. *Incubation period and general course.* Thirteen successful inoculations confirmed by full histological examination were obtained in sheep. In ten of these the virus came from monkeys, and in the remaining three from previously inoculated sheep. As a converse to the conveyance of the disease from the monkey to sheep, the virus was conveyed from a sheep (A. 65, Sh. 3855) to a monkey (A. 66, Monkey 3860), and from this monkey to a series of these animals. All the inoculations were intracerebral under an anaesthetic, and the virus

had been suspended either in 33 per cent. glycerine or in normal saline solution. Also the inoculations were all made within two days of the death of the previously infected host. After an incubation period of three to twelve days the first symptoms of illness were noticed. The duration of the illness until the animal died, or was *in extremis* and killed, ranged from one to five (or seven) days, except in one case which died thirty days after symptoms were first noticed.

The early symptoms were somewhat vague, as might be expected in an animal showing such low mental development. Hanging of the head and disinclination to feed were taken as suspicious; usually there were quiverings of the lips and ears, and sometimes champing of the jaws, suggestive of cud-chewing; occasionally there was dribbling from the mouth, or a mucous discharge from the nose. Later some retraction of the head usually appeared, whilst the animal, still able to stand and walk, tended to circle to one side.

The final stage was sometimes rapid—in one case lasting an hour—and was frequently characterised by convulsions and great respiratory irregularities. Occasionally convulsive seizures occurred earlier in the disease, and the animal temporarily recovered; indeed, some sheep recovered permanently.

The following notes of a case, where death took place soon after the convulsions first occurred, exemplify the early symptoms:

In the morning it was moving about and eating a little. In the afternoon it was seen in the paddock turning slowly in a circle, with its head down as if trying to reach the grass. After making several revolutions it fell on its side and began nibbling, not grass but a small native plant (*Pimelea*) of unattractive appearance. Shortly afterwards its head became retracted and there were slight convulsive movements, whilst the lips and nostrils were trembling and moving irregularly. It was breathing quickly and there were occasional to and fro movements of the forelimbs, less so of the hind limbs. Later the head became markedly retracted, and the limbs rather rigid and partly convulsed. The segments of the hoof of one of the front feet were sometimes widely separated. The animal seemed to be unconscious. More definite convulsive movements occurred occasionally. At the end of one of these, respirations became highly irregular and then ceased, and the animal died an hour after it had been noticed circling round.

2. *Analysis of the symptoms and signs met with in the sheep.* The following summary deals only with the thirteen sheep in which histological examination proved the presence of "X disease." Those that showed slight symptoms, apparently of "X disease," and recovered, are not included.

Convulsive movements or fits, sometimes intense, occurred in eleven of the thirteen sheep. During these attacks the head was thrown back, the limbs were moved convulsively and sometimes pawed the air, and fine twitchings occurred in the lips, nostrils, and ears. In five cases, before the development of more untoward symptoms, the sheep tended to walk in a circle. Quivering or fine tremors of the lips, ears, nostrils, etc., sometimes extending to the whole



body, occurred in nine cases, and were frequently seen, apart from attendant convulsions. Under similar circumstances, champing movements of the jaws were noticed in two cases, and grinding of the teeth in another. Stiff neck or retraction of the head was noticed during the course of the illness in six cases. In two, the legs were rigid or there was a stiff, jerky gait. Protrusion of the tongue occurred in one animal, "staggers" in one, and restlessness in one. One animal was noted as being drowsy. In most cases terminal unconsciousness or coma accompanied the convulsions. Some weakness of the hind legs was seen in one case. In nine instances rapid or irregular breathing occurred during the course of the illness. Sometimes a series of very rapid respirations were followed by a pause, and then the respirations began again. Other interesting signs were running of the nose or a mucous discharge in two sheep, and dribbling from the mouth in two others.

3. *Sheep surviving intracerebral inoculation, but showing slight symptoms, probably of encephalo-myelitis.* A. 82, Sh. 3894. On June 17th this animal was inoculated intracerebrally with the same material as successfully conveyed the disease on the same date to two other sheep, A. 80 and A. 81. Twelve days later the animal seemed ill and was lying down, and was "off its food," but showed no paresis. Beyond these slight symptoms on this date it remained well until July 6th, when blood was taken from it under an anaesthetic. Four days later it died, apparently from the after-effects of the anaesthetic. No histological lesions of the disease were detected.

It is possible that the slight symptoms shown on one day were due to a very mild attack of encephalitis from which the animal recovered, and that the lesions had disappeared by the time the animal died, eleven days later. Under any circumstances the series of experiments to which this case belongs shows that A. 82 possessed a decided relative, if not an absolute, immunity to the disease.

A. 90, Sh. 3903. On June 27th this animal was inoculated intracerebrally with material that gave a positive result in the case of two other sheep, A. 89 and A. 91. On July 2nd it was breathing fast, held its head down, was drowsy and did not eat. On July 3rd it held its head down when resting, was not eating, and did not seem to run about so much or so quickly as usual, while the hind legs seemed to go down easily when pressure was exerted on the back. On July 4th it was still sluggish and not eating, but next day it was well and remained so afterwards.

This animal had been previously inoculated, under the designation A. 68, with material from a positive sheep, which successfully infected the monkey, A. 66, done on the same date. It is possible that the first inoculation, intended to be intracerebral, was made into the frontal sinus, at any rate it was resultless. The sheep had also received a second inoculation, under the designation of A. 86, from Sh. A. 75, which upon histological examination was found not to show the lesions of encephalo-myelitis.



It seems highly probable that the symptoms from which this sheep suffered after the third injection were due to a mild attack of encephalomyelitis from which it recovered. And it can hardly be denied, especially when its previous inoculations are considered, that the animal possessed a relative, if not an absolute, immunity to the virus.

A. 102, Sh. 3914. On July 7th this animal was inoculated intracerebrally with material which successfully conveyed the disease to two sheep A. 103 and A. 105, and to a monkey, A. 100. It became ill on July 12th and had a temperature of 106° F. On July 13th the temperature was lower, 105°, and from then to the 16th it remained well excepting for occasional twitchings and rapid breathing. It seemed, however, very weak and was "gone in the legs," and the nose was running. On July 19th it seemed well.

Blood was taken under an anaesthetic on July 31st. The serum from this blood was used, on August 2nd, for mixing with a virus before its injection into A. 117, M. 3937. This monkey developed the disease twenty-three days later, which is an unusually long incubation period.

The symptoms presented by this sheep suggest that it may have had a mild attack of encephalitis, from which it recovered. This is perhaps supported by the unduly prolonged incubation period resulting in monkey A. 117, which may be possibly attributed to some immunising power possessed by the sheep serum. The course of the disease in the monkey, however, when it developed, was not altered.

A. 125, Sh. 3948. On August 14th this animal was inoculated intracerebrally with an emulsion from a positive sheep, A. 121. Next day it was a little sick, as the result of the operation. On August 18th it seemed cramped and could not use its hindquarters for a few minutes, and then seemed to be convulsed. On August 19th it showed convulsive movements, lasting for about two hours. Thereafter it remained well until August 25th when one hind leg seemed to be slightly contracted and spastic. It was well next day and remained so afterwards.

This sheep had received a previous inoculation intracerebrally on July 7th with material from a monkey, which produced the disease on the same date in two other sheep, A. 103 and A. 105, and in a monkey, A. 100. It had been unaffected by this first inoculation.

It is possible that the symptoms shown by this sheep, A. 125, were due to a mild attack of encephalitis. The result of the first inoculation, however, shows that it must have possessed a marked relative, if not an absolute, immunity to the virus. If the symptoms manifested after the second inoculation were not due to a mild attack of encephalitis, the result would confirm the view that it possessed absolute immunity. Otherwise a relative or varying immunity would be suggested.

A. 130, Sh. 3968. On August 28th this animal was inoculated intracerebrally with material from a monkey which on the previous day had successfully infected A. 129, M. 3967. On September 5th it seemed sick and was not feeding.

It was walking about, the head was drooping and the respirations were rapid. Next day it seemed worse, was "off its food," and was lying down continually, whilst in the afternoon it was circling round with the head somewhat retracted. On September 7th the lips were twitching at times, and it was shivering all over and had a slight limp in one foreleg. On the afternoon of September 8th it seemed worse, the lips were twitching, it kept poking out its tongue, and was circling round and not eating. On September 9th it had two fits, each of about five minutes' duration, during which it lay down, kicked with its fore and hind legs, and seemed unconscious. On September 10th it was still sick but was able to stand. On being made to move it could walk, but occasionally stumbled and tended to fall on one side. From this date it remained well.

This sheep, under the designation of A. 119, on August 2nd had received a previous inoculation, intracerebrally, of a Berkefeld filtrate of monkey material. It had been unaffected by this first inoculation.

The symptoms manifested by this sheep are strongly suggestive of a mild form of encephalitis, from which it recovered. It would appear that the Berkefeld filtrate contained no virus or a sub-infective dose. It is possible that the injection of this filtrate produced some immunity against the second inoculation, leading to the recovery of the sheep, but this is very doubtful.

A. 131, Sh. 3969. On August 28th this sheep was inoculated in the same way and with the same material as A. 130. It had also received a previous inoculation on August 2nd with the same Berkefeld filtrate. On September 5th the animal showed some paresis of the left foreleg, but could run about and was still feeding. The respirations were rapid. Next day it seemed worse. On September 7th it seemed better and could run about and feed. On September 10th it seemed well except for a little stiffness in one hind leg. At no time did it show any twitchings or head retraction.

It is possible, though doubtful, that the slight symptoms shown by this sheep were due to a mild attack of encephalo-myelitis. The remark under A. 130 again applies.

*Comment.* The histories of A. 125 and A. 130 are distinctly suggestive of mild attacks of encephalo-myelitis. Those of A. 90, A. 102 and A. 131 are also suggestive but less so. In the other sheep, A. 82, the result is very uncertain.

If some of these sheep were "mild takes," then there is evidence of bridging between the fatal disease conveyed to some individual sheep and the complete immunity possessed by others.

4. *Inoculation of sheep in series.—Insusceptibility of some animals.* In four instances, series of sheep, five or six in number, were all inoculated at the same time, in the same way, and under the same conditions, with materials consisting of various parts of the brain and spinal cord of successfully inoculated monkeys. The material used varied according to circumstances, but in most cases was a mixed emulsion of various portions of the brain and spinal cord, and in other cases tissue from the frontal and occipital regions only, or from



the medulla alone. In one series the emulsion was treated with sheep sera. In two animals of another series a Berkefeld filtrate of the emulsion was used. The general results obtained from these four series show conclusively that the material might in one individual sheep produce the typical disease, whilst the same material introduced in exactly the same way, at the same time and under the same conditions in another sheep might fail to produce any illness whatsoever. In several instances reinoculations of these apparently immune sheep with material presumably virulent again failed, suggesting, as is indicated in other experiments, that the immunity thus possessed is a real one, and that the occurrence of non-infection in the series was not due to any over-looked inhibitive factor. Therefore it must be considered as established that some individual sheep are susceptible to the virus and develop the disease when the virus is actually introduced into the middle of the cerebrum, while other individual sheep fail to react in any noticeable way after the virus has been so introduced. These results are very important inasmuch as the inference to be drawn from them may, with some reason, be applied to human beings, and may explain why some persons are attacked with the disease while others, apparently equally exposed to the virus, remain unaffected. Hitherto the general opinion as regards "acute poliomyelitis" may be said to have been that many individuals in the community harboured the virus in their naso-pharynx or in some other situation, and that the virus only occasionally gained access to the central nervous system. In other words, it appeared possible that any carrier might contract this disease, provided immunity had not been established, if the organism should gain entrance to the central nervous system. The results of these sheep inoculations show, however, that even after the virus has been introduced into the central nervous system infection may not necessarily result, but that individuals of the species may be absolutely insusceptible to the virus in spite of not having been already immunised to it.

The following is an account of the results of the four series of sheep inoculations mentioned:

(1) A. 80 to A. 85, Sheep 3892 to 3897. The first four of these animals received an inoculation of material from the usual selected areas of the brain and spinal cord; the fifth, material from the frontal and occipital areas of the brain only; and the sixth from the medulla alone. The last-mentioned animal died within three days of the operation, leaving five animals to be accounted for. The sheep inoculated with the frontal and occipital cortex only remained unaffected and later, under the designation A. 109, Sh. 3921, was successfully inoculated with the disease. The inference to be drawn is that in this particular case the material from the frontal and occipital regions of A. 72, Monkey 3873, either did not contain the virus or contained it in a sub-infective amount. Of the four sheep inoculated with the general emulsion, two developed the typical disease, one was unaffected, and one died 23 days after the operation without having shown definite signs of the disease, and without showing the specific histological lesions.



*Summary.* Of four sheep inoculated with the same material, at the same time and under the same conditions, two developed the disease and two did not.

(2) A. 89 to A. 94, Sheep 3902 to 3907. All these animals were inoculated with material from A. 78, Monkey 3890. The first three received the usual mixed emulsion of various parts of the brain and spinal cord; the fourth and fifth, material from the medulla alone; and the sixth material from the frontal and occipital regions only. Of these animals, two of those receiving the general emulsion and one of those receiving the medulla alone developed the disease, while the other three sheep were unaffected.

*Summary.* Neglecting the sheep which received material from the frontal and occipital regions and for which there was no control to show that this material contained the virus, we find that of five sheep in which the virus was introduced into the brain, three developed the disease and two failed to do so.

(3) A. 101 to A. 106, Sheep 3913 to 3918. These animals received an emulsion of the usual parts of the brain and spinal cord of A. 87, Monkey 3900. This emulsion was mixed with normal saline solution for the injections into two sheep; with the serum of a previously inoculated but unaffected sheep for two others; and with the serum of a normal healthy sheep for the remaining two. One of the last-mentioned sheep died in five days before any symptoms were likely to have manifested themselves. The two receiving the emulsion and saline were unaffected, but one of each of the other pairs developed the disease.

*Summary.* Of five sheep in which the virus was introduced into the brain, two developed the disease and three were unaffected.

(4) A. 119 to A. 123, Sheep 3939 to 3943. These animals were treated with material from A. 110, Monkey 3925. The first two received a Berkefeld filtrate and were unaffected; the other three received an emulsion of the usual parts of the brain and spinal cord. Of these, one developed the disease and two were unaffected.

*Summary.* Of three sheep in which the unfiltered virus was introduced into the brain, one developed the disease and two were unaffected.

5. *Sheep showing natural immunity to intracerebral inoculation of the virus.* Eight sheep, viz. A. 63, (76, 93, 115), A. 69 (94), A. 83 (114), A. 101 (125), A. 104 (126), A. 111, A. 122 and A. 68 (86, 90), are shown, in Appendices XX, XXI and XXV, to have been naturally immune to the disease even when the virus was introduced actually into the brain.

## VI. THE DISEASE AS MANIFESTED IN THE CALF.

The disease was conveyed successfully to one calf, as proved by histological examination after death. In two other cases slight symptoms arose, and in one were almost certainly those of encephalo-myelitis. Both of these animals recovered.

The chief symptoms in the calf, in which the presence of the disease was proved, were restlessness and a tendency to "go" in the front legs, followed by a clear discharge from the nostrils, and on the third day by weakness of the legs and a gait which tended to be circular. The illness terminated in death after general convulsive seizures, with rigidity of limbs, muscular tremors, retraction of the head and arching of the back.

## VII. THE DISEASE AS MANIFESTED IN THE HORSE.

Only one inoculation was made into this species of animal and it was successful, as proved by histological examination. The incubation period was nine days. On the first day of illness the animal tended to move towards one side. The next day there were in addition twitchings of the facial muscles, staring eyes and perhaps partial blindness. On the third day the animal was lying down with the head drawn to one side, and irregular movements of the limbs were observed. This was followed by intense and rapid convulsions, during which the animal was unconscious, alternating with short quiescent periods.

## VIII. OTHER EXPERIMENTS AND VARIOUS INFERENCES DRAWN.

1. *The treatment of the virus with various sera before intracerebral inoculation into monkeys.* Four groups of monkeys pass under this category, viz., A. 9 and 10, A. 20 and 21, A. 27 and 28, and A. 116 and 117.

A. 9 and 10. The serum used in the case of A. 9 was pooled from three children who had had infantile paralysis some time previously. In one case infantile paralysis had occurred  $2\frac{1}{2}$  years before, whilst in the other two cases the disease was probably as remote or more so. The children were under treatment, at the Royal Alexandra Children's Hospital, for the residual paralysis. The serum used in A. 10 was obtained from a healthy medical man who had never suffered from infantile paralysis. The usual emulsion of the human virus was mixed with equal amounts of the respective sera, and kept in an incubator for an hour, and then in the ice-chest over-night. Twenty-four hours after the mixing the injections were made.

A. 8 acts as a control to these two monkeys, inasmuch as the untreated emulsion was inoculated intracerebrally the day previously. A. 8 developed the disease nine days after inoculation, and had a length of illness of six days: A. 9 developed the disease twelve days after inoculation, and had a length of illness of eleven days; and A. 10 developed the disease fifteen days after



inoculation, and had a length of illness of three days. The three monkeys were killed *in extremis*.

From these results it would appear that, by the method employed, the sera of cases which had had infantile paralysis two or three years previously were not capable of neutralising the virus of encephalo-myelitis ("X disease"). It is interesting to note, however, that the two monkeys inoculated with the treated virus had a much longer incubation period than the one inoculated with the untreated virus; and as remarkable that A. 10, in which normal serum was used, had a longer incubation period than A. 9, in which pooled sera, wherein protected bodies might have been expected, were used. It may be noted, however, that whilst the disease in A. 10 ran a rapid course after a long incubation period, in A. 9 it ran a very slow course of eleven days, after a somewhat shorter incubation period. It may be further noted that A. 117, another monkey in which the emulsion was treated with a serum, had a phenomenally long incubation period of twenty-three days; on the other hand A. 124, inoculated with an untreated emulsion, had an incubation period of seventeen days.

*Conclusions.* The treatment of the emulsion of the virus with human serum may possibly lengthen the incubation period. Sera obtained from old cases of infantile paralysis were not proved capable of annulling the virus.

A. 20 and 21. These inoculations need not be further discussed, inasmuch as the emulsion employed was obtained from a monkey which histologically showed no evidence of encephalo-myelitis.

A. 27 and 28. The serum employed in the case of A. 27 was derived from a patient who was believed to have had "X disease" in the previous year. The blood had been forwarded by train and the serum kept in an ice-chest for a few days before use. In the case of A. 28, the serum was a normal human one. The method employed was that already described in connection with A. 9 and 10. Both monkeys failed to "take." As there were no control successful "takes" with material from this human case, there is unfortunately no evidence that the virus was present in the emulsion. Therefore nothing can be learned from the experiments.

A. 116 and 117. These monkeys received injections of a virus from A. 110, Monkey 3925. The virus consisted of an emulsion in normal saline solution of tissues from the usual regions in the brain and spinal cord. After being emulsified, the material was centrifuged at a low speed for two minutes, which yielded a supernatant milky fluid and a deposit of coarser fragments. The fluid was divided into two portions, and to each an equal amount of one of the sera to be mentioned was added. The mixtures were shaken, then incubated for two hours, then kept at room temperature for an hour, and finally injected intracerebrally into the respective monkeys. The object of this procedure was to see whether, during the time given and with the amount of serum employed, the infectivity of the virus would be annulled.

A. 116, Monkey 3936, received the supposed virus mixed with serum from



A. 89, Sheep 3902. This sheep was inoculated on June 27th with material from a previous monkey. It showed slight symptoms on July 2nd, 3rd and 4th, which might be considered as indicating a mild form of encephalo-myelitis. On July 5th it seemed well again. On July 18th 20 c.c. of blood were removed under an anaesthetic. It was used for immunity experiments on sheep. The animal was bled again on July 31st under an anaesthetic. Two days later it was sick, apparently from lung trouble, was worse next day and died on August 4th. Histological examination showed lesions of encephalo-myelitis, apparently in a stage of early resolution, suggesting that the symptoms manifested from July 2nd to 4th were due to a mild form of this disease from which the animal was in process of recovery. A. 116, Monkey 3936, remained unaffected by the inoculation of the virus combined with the serum of this sheep.

A. 117, Monkey 3937, received the virus intermixed with the serum of A. 102, Sheep 3914. This sheep had been inoculated on July 7th with material from a previous sheep, A. 101, Sh. 3913. It became ill five days later with a raised temperature, and thereafter had occasional twitchings, breathed rapidly, seemed very weak, was "gone" in the legs, and had a running nose. It appeared perfectly well on July 19th. Blood was withdrawn under an anaesthetic on July 31st. The symptoms manifested by this sheep suggest that it may have had a mild form of encephalo-myelitis from which recovery had resulted. A. 117, Monkey 3937, was found prostrate on the bottom of the cage twenty-three days after the inoculation. It exhibited intense incoördinated movements on being disturbed, and histological examination after death showed typical lesions of encephalo-myelitis.

*Discussion of the results.* First it is necessary to point out that the monkey supplying the virus employed in these two experiments, namely A. 110, Monkey 3925, at the time it was killed appeared as though it might have recovered from the disease. The possibility, therefore, is that the strain of virus at this stage possessed less virulence than it had originally, so that even without any other treatment it might have failed to convey the disease to a monkey, or have only produced the disease after a long incubation period, or in mild form, when it did appear. The results show that A. 116 did not contract the disease and that A. 117 contracted it after an unduly prolonged incubation period of twenty-three days. It is to be noted, however, that when the disease did arise in A. 117, it appeared in an intense form. After making due allowance, therefore, for the possibility that the activity of the virus was waning, the negative result in A. 116 is in support of the view that the serum of A. 89, Sheep 3902, actually did neutralise the virus under the circumstances of the experiment. It might have been expected, moreover, that the serum from this sheep, which was actually shown later to have suffered from encephalo-myelitis, would have possessed immune bodies—if such are developed in this disease—at the time when it was withdrawn. As regards A. 117, the inferences are less clear. The sheep whose serum was employed may or may not have had encephalo-myelitis. The unduly prolonged incubation period may or may not have

been the expression of some immune properties held by this serum. The serum may in fact have delayed the appearance of the disease without modifying its course when it did appear.

2. *The treatment of the virus with various sera before intracerebral inoculation into sheep.* Four sheep, A. 103, 104, 105 and 106, were inoculated with material from A. 87, Monkey 3900. The usual emulsion in normal saline solution was centrifuged at a low speed for about seven minutes. The supernatant fluid was then divided into two portions and each mixed with an equal amount of serum—with serum from a normal sheep, in the case of the last two, and with serum from A. 82, Sheep 3984, in the case of the first two animals. After incubation for two hours, the mixtures were kept at room temperature for a further two hours, and then injected intracerebrally into the respective sheep.

The serum-yielding sheep, A. 82, had been inoculated with material from A. 72, Monkey 3873, on June 17th. On June 29th it seemed ill but showed no paresis. It was bled under an anaesthetic on July 6th for the purpose of the present experiments and died on July 10th, apparently from the after-effects of the anaesthetic. Histological examination showed no lesions of encephalo-myelitis. It should be noted that two companion sheep to A. 82, viz., A. 80 and A. 81, both developed the disease after identical intracerebral inoculations.

A. 103, Sheep 3915, developed the disease, whilst A. 104, Sheep 3916, showed no symptoms, being probably an immune animal.

A. 105, in which the emulsion was treated with serum from a normal sheep, developed the disease, but A. 106, similarly treated, died from lung trouble in five to six days, perhaps before signs of the disease could have manifested themselves.

*Discussion of results.* The serum of A. 82, Sh. 3894, failed to neutralise the virus in the method employed when injected into A. 103, Sh. 3915. As explaining this result, there is no evidence that A. 82, Sh. 3894, actually had encephalo-myelitis, so as to be in the position of possessing anti-bodies in its serum.

The positive results in A. 105, together with the positive results in A. 103, both show that by the method employed sheep serum alone does not neutralise the virus.

A. 111, 112 and 113. As regards these inoculations, the procedure was that adopted in the preceding series, save that the mixtures were incubated for only  $1\frac{1}{4}$  hours and were inoculated an hour later. Again, as showing that the virus was present in the material used, A. 110, Monkey 3925, acts as a control since this received an injection differing only in the replacing of the serum by an equal amount of normal saline solution, and the animal developed the disease. The serum employed for the three sheep was that from A. 89, Sheep 3902, taken on July 18th. A short summary of the history of A. 89 has been already given in discussing the experiments on the two monkeys, A. 116 and



117, in the preceding section. It will be remembered that the serum employed in the case of these monkeys, and which apparently protected A. 116 against infection, was obtained from A. 89, Sheep 3902, on July 31st, whereas the serum employed in the case of these sheep was that obtained on July 18th. A. 111 showed no evidence of encephalo-myelitis after its inoculation; the other two animals died two and four days respectively after the inoculation before any symptoms could possibly have arisen.

*Discussion.* It is possible that A. 111, Sheep 3923, was protected against infection by the blood serum of A. 89, Sheep 3902. As, however, many sheep are naturally immune to the intracerebral injection of the virus, the question of the protective value of this serum at this date is by no means proved.

3. *Experiments suggesting that the virus is no longer present on the 8th or 10th day of illness in monkeys.* In the discussion on the "Failure in Monkeys of Certain Intracerebral Inoculations of Brain and Spinal Cord from Monkeys" (vide Appendix VI) it is suggested that the failure to infect A. 18, Monkey 3823, and perhaps A. 19, Monkey 3824, was due to the disappearance of the virus during the eight or ten days of illness that had elapsed before the death of A. 9, Monkey 3785.

4. *Experiments showing presumed dying out of the virus during prolonged storage in glycerine in the cold.* A. 17, Monkey 3836, was inoculated with material from a monkey on April 11th and was unaffected thereby. The same material on February 13th had successfully conveyed the disease to A. 14, Monkey 3805. That A. 17 was not immune was shown by its successful inoculation later as A. 55, Monkey 3848. The inference is that the virus had died out during the storage for two months in glycerine emulsion in the cold.

A. 96, Sheep 3935 was inoculated on July 29th with material from a monkey, A. 78, Monkey 3890, and remained unaffected. The same material on June 27th and 28th had successfully conveyed the disease to a monkey, a sheep and a horse. This sheep, A. 96, was not further tested to see whether it possessed natural immunity. The failure of the experiment may therefore be attributed either to the length of time the material was preserved in glycerine in the ice-chest, namely a month, or to natural immunity.

5. *Experiments possibly showing a waning in virulence of the virus.* A. 116, Monkey 3936, was inoculated with monkey material which had been treated with the serum of a "positive" sheep, A. 89. The monkey was unaffected by the inoculation, whilst another monkey, A. 117, M. 3937, which was inoculated on the same day with the same material, save the substitution of serum from another sheep, A. 102, developed the disease after an unduly prolonged incubation period of 23 days, the disease thereafter running its usual course. It is probable that the failure of A. 116 to "take" was attributable to the neutralising power of the sheep serum, and it is possible that the prolonged incubation period in A. 117 was due to the presence of similar neutralising bodies, but

to a less degree, in the second sheep's serum. On the other hand it is possible that the negative result in the first monkey, and the prolonged incubation period in the second, were due to a waning in activity of the virus leading to complete failure to "take" in some monkeys and a prolonged incubation period in others. It may be noted that A. 132, which is the same monkey as A. 116, also escaped disease when inoculated later with material from another positive monkey. This second failure may be attributed either to the animal having been rendered artificially immune by the first inoculation, or to the supposed waning of virulence leading to a "take" in some monkeys and a failure in others.

6. *Experiment suggesting the production of artificial immunity.* A. 132, Monkey 3977, which has just been discussed, may be an instance of artificial immunity resulting from the administration of an active virus which had been exposed to the serum of a sheep, which sheep had had encephalo-myelitis and still showed lesions of this disease.

#### IX. SUMMARY AND CONCLUSIONS.

1. The disease is an acute encephalo-myelitis produced by a virus akin to, but not identical with, that of the Heine-Medin disease.

2. The disease was readily communicated, with fatal results, to monkeys (*Macacus rhesus*) by intracerebral inoculation of a suitably-prepared emulsion of nervous substance (brain, cerebellum, pons, medulla and spinal cord) from the human subject dead from "X disease." Moreover, the virus was found to breed true in a succession of thirteen monkey (*Macacus rhesus*) generations.

3. The disease was not communicated to *Macacus cynomolgus* (several trials).

4. The disease was communicated by the above-mentioned method from monkey to sheep (10 times), from sheep back to monkey and on again from monkey to monkey.

5. A certain number of sheep, perhaps 50 per cent., were found wholly insusceptible to the disease; others suffered lightly and recovered.

6. The disease was communicated, with fatal results, by the same method, from monkey to horse (1 case) and to calf (1 case). Two calves suffered lightly after intracerebral inoculation of the usual virus-containing material taken from monkey and horse respectively.

7. The virus appears to be held back completely, or to a great degree, by the pores of a Berkefeld filter.

8. Storage of the virus-containing material in diluted glycerine, under cool conditions, for longer than a few days, reduced or annulled its nocive properties.

9. Drying of the virus-containing material in Petri dishes, in an incubator, probably destroys its activity.



10. In the case of the sheep, there was failure to induce the disease by swabbing the nostrils with virus-containing emulsion.

11. There is some evidence that in the case of the sheep and the calf a previous inoculation with the virus confers immunity.

12. One experiment suggested that artificial immunity might be induced in the monkey by inoculation of virus treated with serum from an "X disease" sheep.

13. Intracerebral inoculation of three dogs, one kitten, two rabbits and one hen failed to produce any signs of the disease; and similar inoculations of two guinea-pigs gave doubtful results.

14. Treatment of the virus-containing emulsion with (a) normal human serum, (b) serum from recovered human cases of "acute poliomyelitis" and (c) serum from "X disease" sheep prolonged the incubation period of the disease in the monkey but did not destroy the virus.

15. Normal sheep serum and serum from "X disease" sheep did not neutralise the virus in its operation on other sheep.

16. Two experiments suggested that the virus was no longer present in the monkey on the eighth or tenth day of illness.

17. Two experiments towards the end of the investigation suggested a waning in strength of the virus.

18. Intraperitoneal and intrasciatic inoculations of virus-containing material, also intracerebral inoculations of cerebro-spinal fluid, of a filtrate of faeces, of a "Noguchi culture," of an emulsion of fowl ticks, of naso-pharyngeal swabs from human cases and contacts, and inoculations into veins, all failed.

## X. APPENDICES<sup>1</sup>.

### APPENDIX I. *Tabulation of the kinds of Inoculations and Results in the respective Species of Animals.*

Each number refers to an individual experiment on an animal. Since in many instances it was necessary, when no result followed, to use the animal again for a further experiment, it is obvious that the number of animals actually used is considerably less than might be inferred from the number of experiments made.

MONKEYS: *Macacus rhesus* and *Macacus cynomolgus* = 62.

Positive results from the intracerebral inoculation of material from three human cases: A. 8, 9, 10, 33, 48 = 5

Positive results from the intracerebral inoculation of material from monkeys: A. 14, 49, 50, 55, 62, 64, 66, 72, 78, 87, 100, 110, 117, 124, 129 = 15

Animals dying shortly after the operation as a direct result of this or from early sepsis. (These cases will not be further considered): A. 7, 13, 22, 25, 47, 51, 128 = 7

<sup>1</sup> Throughout the Appendices the abbreviations M. and Sh. stand for Monkey and Sheep respectively.

Death in 12 days from pathogenic infection, without co-existent evidence of encephalo-myelitis: A. 11	= 1
Failure of intracerebral inoculations of the brain (and spinal cord) from eight human cases: A. 27, 28, 29, 37, 38, 39, 40, 41, 44, 45	= 10
Failure of certain intracerebral inoculations of the brain and spinal cord of monkeys: A. 17, 18, 19, 20, 21, 116, 132	= 7
Failure of intraperitoneal inoculations of human spinal cord: A. 6, 24	= 2
Failure of the intrasciatic inoculation of the brain and spinal cord from a human case: A. 5	= 1
Failure of the intracerebral inoculation of Pasteur-Chamberland F. filtrates: A. 12, 26, 54	= 3
Failure of the intracerebral inoculation of cerebro-spinal fluid: A. 1, 32	= 2
Failure of the intracerebral inoculation of a Pasteur-Chamberland F. filtrate of faeces: A. 31	= 1
Failure of the intracerebral inoculation of a "Noguchi culture"	= 1
Failure of the intracerebral inoculations of naso-pharyngeal swabs from contacts and a case: A. 3, 30	= 2
Failure of the intracerebral inoculation of the brain and spinal cord of a horse: A. 42	= 1
Failure of the intracerebral inoculation of an emulsion of fowl ticks: A. 43	= 1
Failure of the intraperitoneal inoculation of swabs from contacts: A. 2	= 1
Failure of the intrasciatic inoculation of swabs from contacts: A. 4	= 1
Failure of the introduction of the virus into a vein after lumbar puncture: A. 71	= 1

#### SHEEP: = 52.

Positive results from intracerebral inoculation of material from the brain and spinal cord of monkeys: A. 52, 65, 80, 81, 89, 91, 92, 103, 105, 121	= 10
Positive results from intracerebral inoculation of material from the brain and spinal cord of positive sheep: A. 98, 108, 109	= 3
Dying within four and a half days of the operation as a direct result of this, or from post-anaesthetic lung trouble or sepsis: A. 85, 99, 112, 113, 123	= 5
Surviving intracerebral inoculation but showing slight symptoms, possibly of encephalo-myelitis: A. 82, 90, 102, 125, 130, 131	= 6
Showing no symptoms after intracerebral inoculation of brain and spinal cord from human cases: A. 35, 46	= 2
Showing no symptoms after intracerebral inoculation of brain and spinal cord from infected monkeys: A. 53, 63, 69, 75, 83, 84, 93, 94, 96, 101, 104, 106, 111, 122	= 14
Showing no symptoms after intracerebral inoculation of brain and spinal cord from infected sheep: A. 68, 114, 115, 126	= 4
Showing no symptoms after intracerebral inoculation from a monkey histologically negative. (This case will not be further discussed): A. 23	= 1
Showing no symptoms after intracerebral inoculation from a sheep histologically negative. (This case will not be further discussed): A. 86	= 1
Showing no symptoms after swabbing the nose with virus-containing material: A. 70	= 1



- Showing no symptoms after the introduction of horse serum into the spinal canal and of a small quantity of virus into a vein: A. 76 = 1
- Showing no symptoms after intraperitoneal inoculation of the virus: A. 67 = 1
- Showing no symptoms after the intracerebral injection of dried tissue from the brain and spinal cord: A. 77 = 1
- Showing no symptoms after intracerebral inoculation of a Berkefeld filtrate of virus-containing material: A. 119, 120 = 2

## CALVES: = 4.

- Positive result after intracerebral inoculation with material from the brain and spinal cord of a monkey: A. 57 = 1
- Showing symptoms of illness, possibly of encephalo-myelitis, after intracerebral inoculation of material from the brain and spinal cord of a positive monkey or a positive horse: A. 88, 107 = 2
- Showing no symptoms after intracerebral inoculation (a second inoculation) with brain and spinal cord from a positive sheep: A. 127 = 1

## HORSE: = 1.

- Positive result from intracerebral inoculation of material from the brain and spinal cord of a monkey: A. 95 = 1

## DOGS: = 5.

- Showing no symptoms after an intracerebral inoculation of material from the brain and spinal cord of a human case, a monkey case, or a sheep case: A. 16, 34, 56, 97 = 4
- Dying as a result of the operation. (This case will not be further discussed): A. 15 = 1

## KITTENS: = 2.

- Showing no symptoms after intracerebral inoculation of brain and spinal cord from a positive monkey: A. 79 = 1
- Dying as the result of the operation. (This case will not be further discussed): A. 61 = 1

## RABBITS: = 3.

- Showing no symptoms after intracerebral inoculations: A. 58, 73 = 2
- Dying as the result of the operation. (This case will not be further discussed): A. 59 = 1

## GUINEA-PIGS: = 2.

- Showing indefinite symptoms and doubtful histological results after intracerebral inoculation of brain and spinal cord from infected monkeys: A. 60, 74 = 2

## HEN: = 1.

- Showing no symptoms after intracerebral inoculation of brain and spinal cord from an infected monkey: A. 118 = 1.

APPENDIX II. Table of Monkey Inoculations. Positive Results.

No. of Monkey	Date	Source of material	Day of illness when virus was obtained	Hours after death when p.m. examination made	Period virus was in <i>vitro</i>	Menstruum in which virus was preserved	Incubation period in days	Length of illness in days
A. 8, M. 3783	29/1/18	Case 27, Narrabri	3	21	1 day	33% glyc. in saline	9	6
A. 9, M. 3785	30/1/18	" 27 "	3		2 days		12	11
A. 10, M. 3786	30/1/18	" 27 "	3		2 days		15	3
A. 14, M. 3805	13/2/18	A. 8, M. 3783	6	Immediate	1 day	50 % glyc. in saline	10	3
A. 33, M. 3803	13/2/18	Case 38, Wee Waa	2	12	1 day	33 % "	12	6
A. 48, M. 3829	22/3/18	Case 32, Narrabri	5	5 hours	7 days	33 % glyc. sol. in saline	11	3
A. 49, M. 3835	4/4/18	A. 48, M. 3829	3	Immediate	A few hours	Normal saline	7	5
A. 50, M. 3839	15/4/18	A. 49, M. 3835	5	"	"	33 % glyc. sol.	5	5
A. 55, M. 3848	16/4/18	A. 50, M. 3839	5	Some hours	1 day	"	8	2½
A. 62, M. 3845	23/4/18	A. 52, Sh. 3839 b	4	3	1 day	"	5	6
A. 64, M. 3854	7/5/18	A. 55, M. 3848	2½	A few hours	2 days	"	7	5
A. 66, M. 3860	17/5/18	A. 65, Sh. 3855	1	A few hours	1 day	"	6	8
A. 72, M. 3873	30/5/18	A. 66, M. 3860	8	Immediate	A few hours	Normal saline	11	6
A. 78, M. 3890	15/6/18	A. 72, M. 3873	6	"	"	"	10	3
A. 87, M. 3900	27/6/18	A. 78, M. 3890	3	"	"	"	7	4
A. 100, M. 3912	7/7/18	A. 87, M. 3900	4	"	3 hours	"	9	4
A. 110, M. 3925	19/7/18	A. 100, M. 3912	4	"	A few hours	"	11	4*
A. 117, M. 3937	2/8/18	A. 110, M. 3925	4	"	"	"	23	3
A. 124, M. 3952	17/8/18	A. 110, M. 3925	4	"	15 days	33 % glyc.	17	5
A. 129, M. 3967	27/8/18	A. 117, M. 3937	3	"	A few hours	Normal saline	10	4

\* Perhaps would have recovered.

M. = Monkey.

Sh. = Sheep.



APPENDIX III. *Details of the Successful Inoculations in Individual Monkeys (Macacus rhesus).*

A. 8, M. 3783, became ill on the ninth day. During the first two days its movements were violent and incoördinate, and it showed apparent weakness in the legs. On the third day it was quieter, but markedly weak and very clumsy in its movements. On the sixth day, when it was killed, both legs were markedly paretic, whilst the right arm seemed absolutely paralysed and useless.

A. 9, M. 3785, became ill on the twelfth day, showing slight weakness in the legs. Its condition was much the same on the two following days. On the fourth day the right arm seemed decidedly paretic and clumsy, whilst the legs were markedly paretic and dragged after the body on movement. The body swayed on moving and movements were clumsy. On the fifth day the animal was ataxic and frequently fell over, whilst its movements were clumsy and the neck seemed weak. Its condition during the next three days was much the same. On the ninth day, in addition, the head was somewhat retracted; the muscles of both arms and legs still possessed some tone. On the eleventh day, when the animal was killed, it was much exhausted and no movements could be detected in the arms.

A. 10, M. 3786, after an incubation period of fifteen days, showed an anxious expression and seemed drowsy. It reeled like a drunken man; the arms and legs seemed paretic. On the second day of illness it showed marked ataxia and the neck seemed weak. On the third day it was lying on its side; the right arm was apparently paralysed; the left arm and both legs could be moved a little, but some tone still remained in the limbs. The animal was killed on this day.

A. 14, M. 3805, after an incubation period of ten days, became markedly ataxic whilst the hind limbs were apparently paretic. Next day it uttered sharp cries from time to time, and its head was frequently buried on the chest. On being startled it reeled and swayed about in a convulsive way, injuring itself. The hind limbs and right arm were weak. On the third day the animal was quite prostrate; it showed slight convulsive movements of the left arm and left leg, and the head was slightly retracted. The temperature was subnormal. It died on this day.

A. 33, M. 3803, after an incubation period of twelve days, had an anxious expression and showed clumsy movements. There was no definite paresis. On being disturbed, convulsive seizures, without loss of consciousness, occurred. On the second day there was marked incoördination and apparently some rigidity, whilst attacks of convulsive movements occurred from time to time. There was marked incoördination and an occasional twitch of the limbs. Partial paresis of the hands was manifested when it tried to feed itself. The same intense incoördination and general convulsive movements occurred during the next two days. On the fifth day it was lying on its side at the bottom of the cage; both hind limbs seemed useless but rather rigid than flaccid, whilst both arms were paretic. On the sixth day, when it was killed, the animal was still prostrate, and there was paresis of all four limbs which were rather flaccid.

A. 48, M. 3829, became ill on the eleventh day and showed the usual very anxious expression. Its movements were decidedly incoördinate. During the next two days the incoördination was greatly increased and an internal squint was present on the third day of illness when the animal was killed.

A. 49, M. 3835, became ill on the seventh day, showing a frightened expression and being nervous. One arm and leg seemed rather spastic. On the third day of illness it uttered peculiar staccato cries, was very "jumpy," and showed exaggerated incoördinated movements. Next day its condition was worse, and on the fifth day, when it was killed, it was prostrate on the bottom of the cage with the head markedly retracted.

A. 50, M. 3839, showed a frightened expression on the fifth and sixth days after its inoculation. On the third day of illness it uttered barking noises; its movements were exaggerated but there was no definite incoördination. Next day, in addition, there was slight incoördination, and on the fifth day of illness the animal was prostrate on the bottom of the cage, though it could partly raise itself. Its movements were much exaggerated, during which it injured itself. It was killed with chloroform on this day.

A. 55, M. 3848, did not use the left arm on the seventh day after its inoculation. Next day its movements were very shaky and rather incoördinate. This may be considered as the first day of illness. On the second day the animal was intensely shaky, trembling all over as if from paralysis agitans, and was rather incoördinate in its movements; it died during the night.

A. 62, M. 3845, on the fifth day after inoculation appeared to show slight incoördination. Next day its movements were distinctly ataxic, and there appeared to be some weakness in one arm and leg. On the third day it swayed on movement, but the apparent weakness of the arm and leg had disappeared. On the fourth day it was much the same. On the fifth day the hind limbs seemed paretic and movements were very shaky, jumpy, and incoördinate. The animal fell from time to time on the bottom of the cage, and showed violent incoördinated movements, almost convulsive in character. The animal was killed with chloroform.

A. 64, M. 3854, after an incubation period of seven days, seemed jumpy when disturbed. Two days later it looked anxious, and there was marked incoördination of movement. On the fourth day it was lying prostrate on the bottom of the cage. It moved the arms and legs but the movements were incoördinate and paretic. Next day the animal could not sit up or even hold its head up, and the head was slightly retracted. There were slight irregular muscular contractions in the right arm, both legs and the tail. Some tone was still retained in the limbs. Chloroform was administered.

A. 66, M. 3860, after an incubation period of six days, had an anxious expression and its movements were excessive and there was slight incoördination. Next day, in addition, there was weakness of the hind limbs. From the third to the fifth day it was much the same, but on the sixth day the right arm was apparently paretic. On the seventh day it was worse, but could still jump about and feed. On the eighth day it was prostrate on the floor of the cage, and there seemed to be almost complete paralysis. On the eighth day the condition was worse, though the limbs were not absolutely flaccid, and still possessed some tone. The animal was killed by chloroform.

A. 72, M. 3873, after an incubation period of eleven days, had an anxious expression. For the next two days its condition was the same, but on the fourth day there was ptosis of both eyelids, slight incoördination of movement, and dragging of the left leg. On the fifth day its movements were markedly incoördinate and jumpy, and there were slight twitchings of the left arm. The right arm and leg during the day became paretic. On the sixth day it was prostrate on the bottom of the cage, and the right side seemed paralysed. This was found not to be a true complete paralysis, as there was some resistance to passive movement. The knee-jerks and elbow-jerks were present. The animal was killed with chloroform.

A. 78, M. 3890, after an incubation period of ten days, had a slightly anxious expression and seemed more excitable than its healthy fellow. Next day it was markedly ill and apparently intensely sleepy. There was definite ptosis on both sides, but more decided on the left side. It swayed on movement and struggled violently. On the third day it was prostrate, could not raise its head or sit up. There was no head retraction; the limbs still possessed tone. Chloroform was administered.

A. 87, M. 3900, after an incubation period of seven days, had an anxious expression and was rather jumpy. Next day its movements were very incoördinate and it had two convulsions after being disturbed. On the third day its condition was worse, and towards evening it was prostrate on the bottom of the cage, and when disturbed, violent and irregular con-



vulsive movements occurred. On the fourth day it was still prostrate; it could only move the arms and tail a little, but some tone was still present in them. Chloroform was administered.

A. 100, M. 3912, after an incubation period of nine days, had a slightly anxious expression. Next day its countenance was still slightly anxious-looking, and there was decided incoördination and exaggeration of movements. On the third day it had fallen to the bottom of the cage, and could only raise itself partly and then struggled round with convulsive movements. The head became retracted. On the fourth day the animal was still prostrate, uttered occasional barking noises and had head retraction. The limbs were rather rigid and there was no flaccid paralysis though there was probably some paresis. Chloroform was administered.

A. 110, M. 3925, after an incubation period of eleven days, had an anxious expression and walked somewhat "gingerly." Next day there was marked incoördination, and it presented a somnolent appearance at times. There was slight tremor of the arms and occasional tremors of the head, limbs and hands. Next day the head was somewhat depressed on the chest and would gradually sink lower and lower as if the animal were dropping off to sleep, when it would overbalance and struggle incoördinately round. There were slight muscular twitchings, and the eyes were occasionally turned to the left; there was drooping of the eyelids. On the fourth day the monkey seemed distinctly, though slightly, better. There was an inward and upward squint of the right eye, but no ptosis of either lid. It was not now somnolent. Its movements were distinctly incoördinate and violent. There was no definite paresis. The animal looked as though it might possibly recover. Chloroform was administered to obtain the virus.

A. 117, M. 3937, after an incubation period of twenty-three days, was found prostrate on the bottom of its cage. On being disturbed it struggled round with intense incoördinate movements, during which it injured itself. It uttered short sharp barking noises from time to time. It could not sit up or stand, but could grip hold with its hands and feet. On the second day of illness it was quite prostrate but conscious; the right arm seemed paralysed, but it kicked vigorously with both hind legs and the left arm. On the third day of illness, when chloroform was administered, the right arm was severely paralysed though there was still some slight tone in the biceps and very slight tone in the flexors of the forearm. The left arm was markedly paretic and rather rigid, whilst the legs were rather rigid and paretic. The knee and back muscles seemed also paretic. The temperature was subnormal.

A. 124, M. 3952, after an incubation period of seventeen days, was noticed to be slightly "nervous." Next day the left arm and leg were weak, and the animal was still "nervous." On the third day the left arm seemed quite paralysed whilst the left leg and the hind-quarters were weak. It did not seem able to see things properly. On the fourth day it was having convulsions at frequent intervals, lying on the floor of the cage between times. On the fifth day, that in which the animal was killed, the left arm seemed completely paralysed, but it could grip with the right hand and both feet. The temperature was subnormal.

A. 129, M. 3967, after an incubation period of ten days, appeared "nervous" with incoördination of movement and some paresis of the left arm. Next day there was intense incoördination, during which the animal injured itself against the sides of the cage; some of its muscular movements might be described as contortions. On the third day of illness the left arm appeared to be completely paralysed, and there was indefinite weakness of the right arm. Convulsions, lasting a few seconds, occurred from time to time. Incoördination was intense. On the fourth day the animal was found prostrate on the bottom of its cage apparently dead. Its eyes still seemed to recognise its surroundings, however, whilst there were slight indications of movement at the ends of the extremities. The temperature was markedly subnormal.

APPENDIX IV. *Death of a Monkey in Twelve Days from Pathogenic Infection without Co-existent Evidence of Encephalo-myelitis.*

A. 11, M. 3801, was inoculated on February 12th, with material from A. 8, M. 3783, which was killed on this date. A day later A. 14, M. 3805, received an inoculation containing the same virus with the addition of serum from the monkey contributing the virus. A. 14 developed encephalo-myelitis, as proved by final histological examination. A. 11, nine days after inoculation, developed symptoms resembling those of other animals infected with this disease. It died four days later. Histological examination showed a picture obscured by widespread miliary abscesses in which micrococci were detected. There was no histological evidence of encephalo-myelitis. As portions of the brain, from increased intracranial pressure, protruded through the trephined hole, and the surface wound, in consequence, had re-opened after the animal became ill, complicating septic infection was expected. It was, however, believed at the time that the hernial protrusion had been caused by the congestion of the brain due to the development of encephalo-myelitis, and that lesions of this disease would be detected as well as those of the secondary infection; such, however, was not the case. The interesting speculation arose as to whether histological lesions were really present but were overlooked or masked, or whether the pyogenic infection had in some way destroyed the virus of encephalo-myelitis. As against the first of these views, it may be noted that the inoculations made from A. 11 were negative; somewhat in favour of the latter, and as tending to show that active virus was originally injected into A. 11, is the successful result with the same material, used on the following day, in A. 14.

APPENDIX V. *Failure in Monkeys of Certain Intracerebral Inoculations of Human Material.*

Intracerebral injections of emulsions from portions of the brain and (or) spinal cord from human cases failed as follows:

A. 27, M. (*Macacus rhesus*) 3809 (from Case 28, J. M., Narrabri, dying on the sixth day), inoculated with the glycerine emulsion of the upper cervical cord treated with serum of Case 30, O'M, 1917 series, a supposed recovered case. Inoculation made ten days after patient's death.

A. 28, M. (*M. rhesus*) 3810, inoculated as in A. 27, M. 3809, but the glycerine emulsion was treated with a normal serum. Inoculation ten days after patient's death.

A. 29, M. (*M. rhesus*) 3819, inoculated with the same material as A. 27, M. 3809, and A. 28, M. 3810, but without treatment with serum, and twenty-eight days after the death of the patient.

A. 37, M. (*M. rhesus*) 3811 (from Case 6, R. M., Broken Hill, dying on the fifth day of the disease), inoculated with a glycerine emulsion of portions of the brain and spinal cord after transmission through the post. Inoculation eight days after patient's death.

A. 38, M. (*M. rhesus*) 3812 (from Case 7, G. B., Broken Hill, dying on the fourth day of the disease), inoculated with a glycerine emulsion of portions of the brain and spinal cord after transmission through the post. Inoculation eight days after patient's death.

A. 39, M. (*M. rhesus*) 3830 (from Case 18, E. C., Broken Hill, dying on the eighth day of the disease), inoculated with similar material to A. 37, M. 3811, and A. 38, M. 3812. Inoculation eleven days after patient's death.

A. 40, M. (*M. rhesus*) 3828 (from a supposed case in Brisbane). The glycerine emulsion of the brain and spinal cord was inoculated a week or ten days after the patient's death.

A. 41, M. (*M. rhesus*) 3831 (from Case 54, G. S., Narrandera, dying on the 12th day of the disease). Small portions of the brain and spinal cord were transmitted through the post in glycerine, and inoculations made five weeks after the patient's death.



A. 44, M. (*Macacus cynomolgus*) 3825 (from Case 37, A. C. F., Boggabri, dying on the third day of the disease, post-mortem next day). A glycerine emulsion of portions of the brain and spinal cord was inoculated two days after the patient's death.

A. 45, M. (*M. rhesus*) 3837, was inoculated with the same material as A. 44, M. 3825, twenty-nine days after the patient's death.

*Analysis of the above unsuccessful results.*

CASE 28. As regards the experiments contingent on Case 28, J. M., Narrabri, of the two monkeys used for the inoculations, one monkey (A. 28, M. 3810) was two months later successfully inoculated with the disease from A. 50, M. 3839, under the designation of A. 55, M. 3848, on 16/4/18. It would therefore appear that this monkey was susceptible to the disease and that it had not been protected against the later introduction of the virus by having suffered from a mild but unrecognised attack after the first inoculation. Only a very partial post-mortem examination was allowed on this human case, the material obtained being curetted from the upper cervical cord through a small incision in the back of the neck. Failure in the inoculation may be attributed to this particular area of the spinal cord either not containing the virus (though showing the characteristic lesions), or containing the virus in a subinfective amount. The treatment of the emulsions by sera, one of which was normal, cannot be considered as the cause of failure, inasmuch as A. 9, M. 3785, and A. 10, M. 3786, were inoculated with emulsions also treated with sera, and these monkeys "took." It is of course possible that the serum of the supposed recovered case of the previous year might have protected A. 27, M. 3809, had the virus been present. The normal serum, however, should not have protected A. 28, M. 3810. The inoculations were made ten days after the patient's death, and this period of glycerinisation may have been responsible for the failures. As regards A. 29, M. 3819, inoculated with the glycerinated material untreated by serum, twenty-eight days after the patient's death, failure may have again occurred either because of the absence of the virus originally in the material used or on account of the long period during which the virus had been exposed to the influence of glycerine.

*Conclusions.* Failure of inoculations from Case 28, J. M., Narrabri, may be attributed either to the absence, relative or complete, of the virus in the material obtained, or to the length of time, ten to twenty-eight days, during which the glycerinated material was kept.

*Broken Hill and Narrandera Cases.* As regards the four cases in which the material was transmitted by post, we did not take this material ourselves, and it is possible that portions of cerebral tissue containing the virus were not selected. The material came in blocks in glycerine during the warm period of the year, and exposure to the late summer high temperature may have destroyed the virus. The inoculations were made in two instances eight days after death of the host, in one eleven days, and in one five weeks. As the patients died on the 4th, 5th, 8th and 12th days of the disease the virus should have been still present in the brain or cord of two of these cases at least, when death occurred.

*Conclusions.* Failure in these four cases may be attributed to the exposure of the glycerinated material during transit for several days to the high temperature of late summer—a condition inimical to the keeping qualities of vaccinia virus for instance—or to the period of exposure to glycerine after death of the patient, which was eight days or longer; or possibly to infective material not having been selected.

*Brisbane Case.* As regards the Brisbane case, the material was obtained by a colleague, Dr Bradley, so that portions likely to contain the virus were selected. The glycerinated material was necessarily kept at late summer temperature during transit, and was inoculated a week or ten days after the death of the patient.

*Conclusions.* Failure may be attributed to the elevated temperature during transit and the long period before the inoculation was made.

CASE 37. As regards the material from Case 37, A. C. F., Boggabri, the first monkey inoculated was *M. cynomolgus*. We have not so far obtained a successful result in the few inoculations we have made into this species of monkey. The patient died on the third day of the disease; the material was taken next day, and the monkey (A. 44, M. 3825) was inoculated on the succeeding day. A. 45, M. (*M. rhesus*) 3837, was inoculated twenty-nine days after the patient had died; that it was not immune to the virus was shown by a successful inoculation six weeks later (A. 72, M. 3873).

*Conclusions.* Whilst the failure of A. 45, M. 3837, to "take" might be attributed to the length of time that had elapsed after the patient had died, there seems no explanation, except perhaps the species of monkey employed, for the failure of A. 44, M. 3825. The material used in the glycerine emulsion was obtained from the frontal, parietal, occipital and temporo-sphenoidal regions of the cerebrum, and from the cerebellum, pons, medulla, and cervical, dorsal and lumbar regions of the cord—that is from parts which have been successfully employed in other cases. The child died on the third day of the disease before the virus could be expected to have died out. A post-mortem was made on the day after death—a period which allowed successful results in other cases. The first inoculation was made only two days after death—that is, after a period which gave success in other cases.

#### APPENDIX VI. *Failure in Monkeys of Certain Intracerebral Inoculations of Brain and Spinal Cord from Monkeys.*

A. 17, M. 3836, failed to develop the disease when inoculated on April 11th with material from A. 8, M. 3783, which material, when used on February 13th, conveyed the disease to A. 14, M. 3805. That A. 17 was not immune was shown by its successful inoculation later as A. 55, M. 3848. The inference is that the virus died out during its storage as a glycerine emulsion for two months.

A. 18 and A. 19. These two experiments are discussed under the sections dealing with the influence in monkeys of the treatment of the emulsion of the virus with various sera. Though the serum used in the case of A. 19 may have afforded protection, the "normal" human serum used in A. 18 cannot be expected to have done so in this case. The source of the supposed virus used in the experiments on A. 18 and A. 19 was A. 9, M. 3785, which had an unusually long incubation period of eleven to thirteen days, and a prolonged illness of ten or eight days. It is possible that, by the time this monkey was killed, the virus causing its disease had died out, or was only present in subinfective amount. This would explain the failure in both monkeys.

A. 20 and A. 21. The failures in these monkeys are easily explained, inasmuch as there was no histological evidence that the source of the virus, A. 11, M. 3801, had the disease at the time of its death.

A. 116. The failure in this monkey, when its fellow, A. 117, "took," is fully discussed in the section dealing with the influence of various sera on the virus. The evidence suggests that it was protected against infection by the serum of A. 89, Sh. 3902, which had had, and perhaps still had at the time of its death, the disease in question. Another explanation of the failure is that the strain employed had undergone a phase of weakening in virulence, through which the virus, on this account alone, failed to infect certain individual monkeys. Still another explanation is that the monkey was naturally immune, or had been rendered artificially immune by a previous intracerebral injection of material capable of causing immunity but not of producing the disease.

A. 132. This monkey is the same animal as A. 116. It failed to take when inoculated with material two days old from A. 129, M. 3967, which might reasonably have been expected to convey the disease. It is reasonable to suppose that artificial immunity had been



established in this monkey by the inoculation of the virus combined with a (presumed) immune serum, referred to under A. 116, or possibly by the first intracerebral inoculation.

*Summary.* Reasonable explanations are forthcoming for the failures of all these inoculations.

#### APPENDIX VII. *Failure of Intraperitoneal Inoculations.*

Intraperitoneal inoculations failed in animals as follows:

A. 2, M. (*Macacus rhesus*) 3777. Swabbings of the naso-pharynx were made from several contacts of Case 26, J. C. B., Narrabri, and the swabs were emulsified in a glycerine solution. The patient had died on January 15th, a day before these swabs were taken; the glycerine emulsion was inoculated on January 17th.

A. 6, M. (*M. rhesus*) 3781. From Case 27, A. B., Narrabri, dying on the third day of the disease. Post-mortem examination next day. Inoculation made two days after the patient's death with material from the brain and spinal cord.

A. 24, M. (*M. rhesus*) 3798. From Case 28, J. M., Narrabri, dying on the sixth day of the disease. Post-mortem examination next day. A glycerinated emulsion of the upper part of the cervical cord inoculated three days after the patient's death.

A. 67, Sh. 3861. From A. 65, Sh. 3855, dying with typical lesions of this disease. The glycerine emulsion of the brain and spinal cord inoculated two days after death.

*Analysis of Results.* From A. 2, M. 3777, inoculated from the swabbings of contacts of a case of the disease, nothing is to be learned.

The failure of A. 6, M. 3781, is important, inasmuch as A. 8, M. 3783, inoculated intracerebrally on the same day, and A. 9, M. 3785, and A. 10, M. 3786, inoculated intracerebrally on the next day—all took.

The failure of A. 24, M. 3798, must be considered in connection with the failures of A. 27, M. 3809, A. 28, M. 3810, and A. 29, M. 3819, injected intracerebrally. The period after death of the host at which the inoculation took place in this monkey was only two days. Even had infective material been present it is possible that, as in the case of A. 6, M. 3781, the monkey might not have taken.

A. 67, Sh. 3861, failed to take intraperitoneally, whilst the same material inoculated on the same day into A. 66, M. 3860, took intracerebrally. That this sheep was not naturally immune is shown by its successful inoculation six weeks later (A. 89, Sh. 3902).

*Conclusions.* A. 6, M. 3781 and A. 67, Sh. 3861, show that infective material injected intraperitoneally may fail to convey infection, whilst the same material injected intracerebrally may be successful.

#### APPENDIX VIII. *Failure of Intrasciatic Inoculations.*

A. 4, M. 3779, was inoculated in this way with an emulsion of the throat swabbings from the contacts of a case. The animal was *Macacus cynomolgus*—a species in which, in the few inoculations made into it, we have been so far unsuccessful in producing the disease. Further, we do not know as yet whether the virus is frequently present, or present at all, in the naso-pharynx of contacts or cases.

A. 5, M. 3780, was injected with an emulsion which "took" on the same date by intracerebral inoculation in the case of A. 8, M. 3783, and a day later by the same route in A. 9, M. 3785, and in A. 10, M. 3786.

#### APPENDIX IX. *Failure of Pasteur-Chamberland F. filtrates.*

All these inoculations were negative. To enable the material to pass through the filter it was necessary to dilute it to a considerable extent.

A. 26, M. 3807, need not be further considered inasmuch as the inoculation of other material from the same case failed to "take," and hence the presence of the virus in the material as used was not established.

A. 12, M. 3802, was inoculated with a filtrate from the ten times diluted emulsion of A. 8, M. 3783, on the day of this animal's death. As A. 14, M. 3805, inoculated next day with the same emulsion, diluted with an equal amount of monkey serum, developed the disease, the virus was manifestly present in the emulsion before its filtration.

A. 54, M. 3846, was inoculated with a filtrate obtained from an emulsion from A. 50, M. 3839, on the day of this animal's death. As A. 55, M. 3848, inoculated next day with the untreated emulsion, developed the disease, the virus was manifestly present in the original emulsion.

*Conclusion.* The diluted virus has been proved in two cases not to pass through the pores of a Pasteur-Chamberland F. filter, at least in sufficient quantity to produce infection in monkeys by the intracerebral injection of about 1 c.c. of filtrate.

#### APPENDIX X. *Failure of Cerebro-spinal Fluid to Cause Infection.*

A. 1, M. 3776, was unaffected by the intracerebral injection of cerebro-spinal fluid taken from a fatal case on the second day of illness and injected two days later.

A. 32, M. 3800, inoculated intracerebrally four days afterwards with cerebro-spinal fluid taken from a fatal case on the third day of illness, failed to take.

M. 3689, was inoculated intraperitoneally and intrathecally with cerebro-spinal fluid from Case 28, F. R., of the 1917 series.

#### APPENDIX XI. *Failure of a Pasteur-Chamberland F. Filtrate of Faeces.*

A. 31, M. 3779, received an intracerebral injection of a Pasteur-Chamberland F. filtrate of faeces obtained from Case 36, J. K., Boggabri. It remained unaffected. As failures have resulted from the use of such Pasteur-Chamberland F. filtrates obtained from material known to be virulent, the failure in this case teaches us nothing. Attention may also be called to the fact that the monkey used was *Macacus cynomolgus*, a species to which we have so far not been able to convey the disease.

*Summary.* The failure of this filtrate leaves open the question as to whether the virus may or may not be present in the faeces.

#### APPENDIX XII. *Failure of a "Noguchi Culture."*

An attempt was made to grow the virus from monkey material according to Noguchi's method for spirochaetes (*Jnl. Exp. Med.*, XVI, 1912, p. 621).

A diffuse cloud appeared in one of the original cultures which otherwise remained sterile, and this material was inoculated a month after the death of the monkey from which it was obtained. The animal remained perfectly well, and was later successfully inoculated with further material.

*Summary.* Failure resulted from the inoculation of a first generation of a presumed Noguchi culture a month after the death of the monkey from which it was made.

#### APPENDIX XIII. *Failure in Monkeys of Intracerebral Inoculations from the Nasopharyngeal Swabs of Contacts and of a Case.*

A. 3, M. 3778, was inoculated with an emulsion in glycerine solution of the nasopharyngeal swabs of several contacts of a case, and A. 30, M. 3797, with a similar swabbing from an actual case at the height of the disease. These experiments were conducted on the same animal, which happened to be *Macacus cynomolgus*, a species to which, in the few experiments we have made, we have so far been unsuccessful in conveying the disease. Apart from this, the method adopted was not one that entailed any concentration of the virus. These experiments, therefore, neither prove nor disprove the possible presence of the virus in the nasopharynx of contacts or cases.



APPENDIX XIV. *Failure in a Monkey of the Intracerebral Inoculation of Brain and Spinal Cord from a Horse.*

A. 42. This failure throws no light on the present disease. A horse died at Narrabri from a nervous complaint, which has not been shown to be connected in any way with human encephalo-myelitis.

APPENDIX XV. *Failure of Intracerebral Inoculation of an Emulsion of Fowl Ticks (Argas persicus).*

A. 43. The failure in this monkey throws no light upon the disease in question. The reason why an inoculation was made of an emulsion of fowl ticks is discussed in the section on the possibility of the occurrence of an intermediate (invertebrate) host of the virus in our full official report. It was thought possible that the human encephalo-myelitis might be due to some parasite transmitted by fowl ticks, such as the spirochaete producing the spirochaetosis of these birds.

The failure of the monkey does not support any such contention, though it does not necessarily exclude it.

APPENDIX XVI. *Failure in a Monkey and a Sheep after the Introduction of Horse Serum into the Spinal Canal and Introduction of the Virus into a Vein.*

The animals thus dealt with comprise A. 71, M. 3872, and A. 76, Sh. 3877. The virus in each instance was obtained from monkeys. In the case of A. 71, M. 3872, the virus had been kept for nine days, and, though no positive results were obtained from other inoculations of this virus, there is every reason to consider that it was present when the monkey yielding it was killed. In the case of A. 76, Sh. 3877, the virus was a day old, and had produced a successful result by intracerebral inoculation on the previous day in A. 72, M. 3873. The amount of the virus introduced, about 0.5 c.c., may have been too small to produce infection by this route in either animal. Further, as regards the monkey inoculation, the virus may have died out during the nine days in which it was kept in an ice-chest; and, as regards the sheep inoculation, the sheep employed is shown, under the designation A. 93, to have been naturally immune to the disease. These experiments, therefore, neither prove nor disprove the possibility of causing infection by the method employed.

APPENDIX XVII. *Table of Sheep, Calf and Horse Inoculations. Positive Results with Death.*

No.	Source of virus	Date of inoculation	Incubation period in days	Days of illness at death
A. 52, Sh. 3839 <i>b</i>	A. 49, M. 3835	16/4/1918	3	3
A. 65, Sh. 3855	A. 55, M. 3848	7/5/1918	6	3
A. 80, Sh. 3892	A. 72, M. 3873	17/6/1918	7	5
A. 81, Sh. 3893	A. 72, M. 3873	17/6/1918	12	4
A. 89, Sh. 3902	A. 78, M. 3890	27/6/1918	5 or 7	30 (death adventitious?)
A. 91, Sh. 3904	A. 78, M. 3890	27/6/1918	6	1
A. 92, Sh. 3905	A. 78, M. 3890	27/6/1918	4 (or more)	5 (or less)
A. 98, Sh. 3910	A. 92, Sh. 3905	6/7/1918	5	5
A. 103, Sh. 3915	A. 87, M. 3900	7/7/1918	9 (or 7)	2 (or 4)
A. 105, Sh. 3917	A. 87, M. 3900	7/7/1918	7 (or less)	3 (or more)
A. 108, Sh. 3920	A. 98, Sh. 3910	16/7/1918	6	1
A. 109, Sh. 3921	A. 103, Sh. 3915	18/7/1918	9 (possibly 6)	1 (possibly 4)
A. 121, Sh. 3941	A. 110, M. 3925	2/8/1918	5 or 7	7 or 5
A. 57, Calf 3848 <i>b</i>	A. 50, M. 3839	25/4/1918	5	3
A. 95, Horse 3908	A. 78, M. 3890	28/6/1918	9	3

APPENDIX XVIII. *Summary of Successful Inoculations in Sheep, a Calf and a Horse.*

The foregoing table (Appendix XVII) indicates the source of the virus (whether from a monkey or a sheep), the date of inoculation, the incubation period and the duration of the illness. In those cases in which previous inoculations had been made, details will be found in the section dealing with sheep reinoculations.

A. 52, Sh. 3839 *b*, after an incubation period of three days, became slightly sick and seemed to have difficulty in getting its head down to nibble grass. Next day the animal was restless; its legs seemed somewhat rigid; there was stiffness in the neck, and the head was turned to one side; the lower jaw quivered, the ears twitched and the animal tended to walk in a circle, and took fits during which the head was thrown back and it pawed the air. It presented much the same symptoms on the next day and was unconscious on the third day of illness when it died—exactly six days after the inoculation.

A. 65, Sh. 3855, became ill after an incubation period of six days. The symptoms consisted of shallow and fairly rapid respirations, some stiffness of the neck, quivering of the nostrils and a somewhat slow gait. On the second day it was very sick, would jump round, fall on the ground and struggle, and had convulsive movements. It died on the morning of the third day of illness.

A. 80, Sh. 3892, after an incubation period of seven days, became ill, holding its head down and lying down most of the time. Next day its lips were quivering and the head was turned to one side. On the fourth day it was dribbling from the mouth; there was no apparent paresis. On the fifth day it was lying on its side with the head thrown slightly back and had frequent convulsive movements. At this stage it was killed.

A. 81, Sh. 3893, became ill on the twelfth day. It ran holding its head high and breathing quickly. The ears and lips were twitching slightly. Its temperature was 106° F. Next day, in addition, the animal tended to circle round holding its head high. On the fourth day it was lying on its side with the head somewhat retracted. It manifested convulsive movements from time to time and twitched all over.

A. 89, Sh. 3902, showed, on the fifth day after inoculation, rapidity of breathing, protrusion of the tongue, and running from the nose. Next day it was still breathing quickly. On the seventh day it fell down on one occasion and struggled, and later held its head high and made "champing" movements of the head, lips and jaws. On the eighth day it seemed well again. Twenty-one days after the inoculation it was bled, and it was bled again thirteen days later. Two days after the last bleeding it apparently had respiratory trouble, became worse next day, and died thirty-eight days after the inoculation. Typical histological changes were present.

A. 91, Sh. 3904, on the second and third days (excluding the day of inoculation) seemed ill, the symptoms suggesting some respiratory trouble following the anaesthetic. On the fourth and fifth days it was better. On the sixth day it was not feeding; it turned slowly in a circle and then fell on its side with retraction of the head and trembling of the lips and nostrils. Convulsions then supervened and the animal seemed unconscious. The respirations were highly irregular. It died an hour after the manifestation of these grave signs.

A. 92, Sh. 3905, on the fourth to the sixth day after inoculation seemed ill, and on the last of these days had a mucous discharge from the nose. On the seventh day its temperature was 105° F. and the respirations were sometimes rapid. When placed on its legs it moved backwards and to one side with a stiff and jerky gait. It was lying down most of the time with the ears twitching. Next day it was much the same. There was a mucous discharge



from the nostrils and very irregular respirations, and occasionally convulsive movements. The temperature reached 106° F. It died early next day.

A. 98, Sh. 3910, became ill on the fifth day. On the sixth day the temperature was 107.4° F. It was drowsy and the breathing was rapid. On the eighth day it tended to circle towards one side and ran into objects, and was breathing rapidly. On the ninth day it showed convulsive movements all day, and died during the night.

A. 103, Sh. 3915, had a temperature of 107° F. on the sixth day but did not show definite symptoms until the ninth day. Its temperature now was 104°. It showed occasional twitchings and was breathing rapidly, and died on the tenth day.

A. 105, Sh. 3917, had a temperature of 106° F. on the fifth day. On the seventh day it was dribbling from the mouth and hardly moved. On the eighth day it had a convulsion and there seemed to be weakness in the hind legs. On the ninth day there were continuous twitchings of the nose and mouth, and it died during the following night.

A. 108, Sh. 3920, six days after inoculation, became sick and had rapid respirations. The same evening it exhibited "staggers," and died during the night.

A. 109, Sh. 3921, between the second and eighth days showed occasionally rapid breathing, but no other definite symptoms. It became definitely ill on the ninth day. The head was retracted, the animal circled and showed twitchings of the lips. These were followed by convulsive movements and death.

A. 121, Sh. 3941, on the fifth day after inoculation, was breathing fast. Next day it was not taking its food and seemed weak and the temperature was raised. Convulsive movements appeared on the seventh day after inoculation and occurred again the following day. On the ninth day it had intense convulsive movements lasting for about an hour, together with fine quiverings of the ears and eyelids, twitchings of the head and working movements of the jaws, and very irregular breathing. The animal seemed unconscious during the convulsive attacks. When these had passed off the animal could only stand by leaning against some support, and in walking it swayed from side to side dragging one hind leg a little. Next day the head was retracted and the back markedly arched. Respirations were irregular and there was continual grinding of the teeth. On the eleventh day after inoculation it was lying on its side. The head was slightly retracted; the tail and back legs moved on stimulation. The animal died in the afternoon.

A. 57, Calf 3848 *b*, first showed symptoms five days after inoculation, when it kept its head dependent, inclined to "go" in the front legs, and was restless. Next day there was a clear discharge from the nostrils. On the third day of illness the animal was walking about, the legs were weak and the gait tended to be circular. General convulsive seizures then developed, accompanied by rigidity of the limbs and muscular tremors, whilst the animal lay on its side with the head markedly retracted and the back arched. It was possibly unconscious. After the convulsions had continued for about twenty minutes the animal became fairly quiet, but died an hour later. Typical lesions were found on histological examination.

A. 95, Horse 3908, on the ninth day after inoculation had two seizures, during which it walked round towards the left. Next day its head was depressed, and on moving it tended to "go" towards the left side and threatened to fall on this side. There were twitchings of the facial muscles, staring eyes and apparently partial blindness. On the third day (of illness) the animal was lying on its right side with the head drawn to the left and manifested irregular movements of the left fore and hind limbs. The nostrils were working and the mouth at times was drawn to the left side. Later, intense and repeated convulsions developed alternating with short quiescent periods, and at this stage the animal was killed. Histological examination showed typical lesions.

APPENDIX XIX. *Sheep showing no Symptoms after an Intracerebral Inoculation of Brain and Spinal Cord from Human Cases.*

A. 35. On February 26th this sheep was inoculated intracerebrally with material from a human case which infected A. 33, M. 3803, when similarly inoculated on February 13th. Later this sheep, under the designation A. 65, was successfully inoculated from a monkey. This shows that the failure in the first inoculation was not due to natural immunity, and the inference therefore is that the virus had died out during the thirteen days of its storage in an ice-chest between the time of the successful inoculation of the monkey and its use on this sheep.

A. 46. This happens to be the same sheep as A. 35, and as is thereunder indicated it was finally successfully inoculated under the designation A. 65, so that it clearly possessed no natural immunity to the disease. The first inoculation was made on April 16th with material from Case 37, A. C. F. (J.). As previous inoculations of this material on March 15th and April 11th into monkeys were both failures there is no evidence that the virus was present in the materials used at the time of any of these inoculations.

*Comment.* There are reasonable explanations for these failures.

APPENDIX XX. *Sheep Showing no Symptoms after the Intracerebral Inoculation of Brain and Spinal Cord from Infected Monkeys.*

A. 53 and A. 84. The failure in these experiments is discussed in the section dealing with reinoculations. In these animals later inoculations were successful, and reasonable explanations are given for the initial failures.

A. 63 (93), A. 69 (94), A. 83, A. 101, A. 104. These experiments are also discussed in the section dealing with reinoculations. The evidence indicates that these sheep were naturally immune to the disease.

A. 75. Death occurred on the fifth day, apparently from lung trouble. No histological lesions of encephalo-myelitis could be detected though convulsive movements on the fourth day suggested the possibility of a "take." As the incubation period in some sheep has been apparently as short as three days, it is possible that this animal really did have the disease, but died before recognisable histological changes developed.

A. 96. The inoculation in this sheep was made with material preserved for over a month in glycerine in an ice-chest and obtained from a monkey. In the fresh state, this virus conveyed the disease to a monkey, a sheep and a horse. Failure may be attributed either to the length of time the material was preserved or to natural immunity.

A. 106. This animal died from post-anaesthetic lung trouble five to six days after inoculation. No evidence of the disease was detected histologically, though it might or might not have had time to develop. This sheep may have been naturally immune.

A. 111. Since the same material, as was used in this experiment, was successful on the same day in conveying the disease to A. 110, M. 3925, the failure is to be attributed to a natural immunity.

A. 122. The successful results in A. 117, M. 3937, and in A. 121, Sh. 3941, on the same date with the same material, indicate that natural immunity is the explanation of the failure in A. 122.

*Summary.* In seven of these sheep, natural immunity seems clearly to be the explanation of the failures. In two, death occurred so early, from complications, that the disease, if about to develop, may not have had time to manifest itself or produce recognisable lesions. In one, the length of storage of the virus may have destroyed its activity. In the other two, reasonable explanation of the failures are given elsewhere.



APPENDIX XXI. *Sheep showing no Symptoms after the Intracerebral Inoculation of Brain and Spinal Cord from Infected Sheep.*

A. 68, A. 114, and A. 115. These three animals are all discussed in the section dealing with re-inoculations, where it is shown that they were naturally immune.

A. 126. This animal failed to take definitely whilst its fellow, A. 125, showed symptoms, possibly due to encephalo-myelitis, and recovered. A. 126 may have enjoyed natural immunity.

APPENDIX XXII. *Failure in a Sheep of an Intracerebral Inoculation of Dried Brain and Spinal Cord from a Positive Monkey.*

A. 77, Sh. 3878, received an intracerebral injection of emulsified dried brain and spinal cord from a positive monkey eight days after this monkey's death. The material had been dried in Petri dishes in an incubator.

As many sheep are immune to intracerebral inoculation of the virus, the failure in this case neither proves nor disproves the possibility of the virus resisting desiccation for eight days at incubator temperature.

APPENDIX XXIII. *Failure in Sheep of a Berkefeld Filtrate from a Positive Monkey.*

A. 119, Sh. 3939, and A. 120, Sh. 3940, received intracerebral injections of a Berkefeld filtrate and remained unaffected, whilst inoculations on the same date of the unfiltered emulsion were successful in conveying the disease to A. 117, M. 3937, and A. 121, Sh. 3941.

One of these sheep, A. 119, Sh. 3939, received a further inoculation twenty-six days later, which was followed a week later by symptoms suggesting slight encephalo-myelitis from which the animal recovered. There is reason therefore to think that this sheep was not naturally immune.

The inference to be drawn from these experiments is that the virus did not pass through the pores of the Berkefeld filter, at least in sufficient quantity to induce infection.

APPENDIX XXIV. *Failure to Convey the Disease to a Sheep by Intranasal Swabbing.*

A. 70, Sh. 3865, had the inside of the nostrils vigorously swabbed with an emulsion of the brain and spinal cord of A. 64, M. 3854 and was unaffected thereby. A. 69, Sh. 3864, inoculated intracerebrally with the same material, on the same date, failed to develop the disease. A. 71, M. 3872, seven days later had an injection of horse's serum into the spinal canal and a small quantity of the material from this monkey (A. 64) introduced into a vein. It also remained well. These were the only three animals receiving inoculations from A. 64, M. 3854, and none of them developed the disease. Only one was inoculated intracerebrally, the only route by which we have so far obtained infections, and the animal so inoculated was a sheep, a species which we have found frequently to be immune to the virus introduced by this route. That A. 70 was not immune to the virus was shown later, under the designation A. 92, Sh. 3905, when the disease was successfully conveyed to it.

*Comment.* The result of this experiment neither proves nor disproves the possibility of infection occurring through the nose.

APPENDIX XXV. *Sheep Reinoculations.*

For various reasons—economy of expensive animals and testing for natural immunity being the most important—a number of sheep were reinoculated. The animals so dealt with were fourteen in number. They may be divided into those which "took" after reinoculation

and those in which reinoculations were unsuccessful. Unless otherwise stated, the inoculation was intracerebral and the material used an emulsion of portions of the brain and spinal cord. There were six in the first and eight in the second group.

(a) *Successful Reinoculations.*

A. 23, Sh. 3816—A. 52, Sh. 3839 *b*. The first inoculation was made on February 26th with material from A. 11, M. 3801. It was afterwards found by histological examination that this monkey did not show the lesions of encephalo-myelitis. The later successful result by the inoculation on April 16th can therefore be explained.

A. 35, Sh. 3817—A. 46, Sh. 3839 *c*—A. 65, Sh. 3855. The first inoculation was made on February 26th with material from Case 38, G. H. From this human case A. 33, M. 3803, had been successfully inoculated on February 13th. The second inoculation was made on April 16th with material from Case 37, A. C. F. (J.); A. 44, M. 3825, was unsuccessfully inoculated on March 15th and A. 45, M. 3837, on April 11th with the same material. The successful result of the inoculation on May 7th from a monkey, A. 55, M. 3848, may be explained in connection with the first inoculation by the view that the glycerinated virus may have died out in the interval of a fortnight that elapsed between the successful inoculation in the monkey and the unsuccessful one in this sheep. As regards the second inoculation there was no evidence by the two monkey inoculations that the virus was present in the material used.

A. 53, Sh. 3863—A. 91, Sh. 3904. The first inoculation was made with material which, though originally infective, as shown by positive results in A. 50, M. 3839, and A. 52, Sh. 3839 *b*, had been conserved in the ice-chest for a month. The later positive result is therefore best explained by supposing that the first virus had died out during the period of its storage.

A. 67, Sh. 3861—A. 77, Sh. 3878—A. 89, Sh. 3902. The first inoculation on May 17th was an intraperitoneal one of an emulsion from a positive sheep, A. 65, Sh. 3855. The second experiment was on June 7th, and consisted of the intracerebral injection of dry brain material from A. 66, M. 3860. The third inoculation was made on June 27th from A. 78, M. 3890. A few days after the last inoculation it seemed sick and presented a few slight symptoms, possibly indicating a mild form of the disease. On July 18th blood was taken under an anaesthetic and it was bled again similarly on July 31st. It died on August 4th, apparently from lung trouble as a result of the anaesthetic, but histological examination showed the lesions of encephalo-myelitis, possibly in an early stage of resolution. Serum from the blood taken on the first occasion may have protected A. 111, Sh. 3922. It certainly seems as though the second sample of blood, when mixed with the virus before injection, protected A. 116, M. 3936.

In connection with the first injection, which was into the peritoneum, we possess as yet no evidence that this is a successful route for introducing the virus. As regards the second inoculation which was with dry brain material, the drying may reasonably be considered as having destroyed the virus. This animal cannot therefore be considered to have been proved to be naturally immune to the disease before the third inoculation with its delayed successful result.

A. 70, Sh. 3865—A. 92, Sh. 3905. The first experiment, performed on May 20th, consisted in swabbing the nose with an emulsion from A. 64, M. 3854. The successful result, therefore, of the intracerebral inoculation on June 27th can be explained by the first procedure not having been a reliable method for obtaining infection.

A. 84, Sh. 3896—A. 109, Sh. 3921. The first inoculation was made with tissues from the frontal and occipital regions only of the monkey yielding the virus. The general emulsion of the brain and spinal cord of this monkey gave positive results in A. 78, M. 3890; A. 80, Sh. 3892; and A. 81, Sh. 3893. The second inoculation, which was successful, was with the



mixed emulsion of brain and spinal cord from an infected sheep. The failure of the first inoculation may be explained by absence, relative or complete, of virus in the material used.

(b) *Unsuccessful Reinoculations.*

A. 63, Sh. 3853—A. 76, Sh. 3877—A. 93, Sh. 3906—A. 115, Sh. 3934. The first inoculation was made on May 3rd from a positive monkey, A. 62, M. 3845. The second experiment, performed on May 31st, consisted of lumbar puncture with the intraspinal injection of 2 c.c. of antimeningococcal serum, together with an injection into a vein of 0.5 c.c. of virus from A. 66, M. 3860. The third experiment, on June 27th, consisted of the intracerebral injection of a saline emulsion of the medulla of A. 78, M. 3890. The companion sheep, A. 92, Sh. 3905, similarly injected at the same time, gave a positive result. The fourth inoculation was made on July 29th from a positive case in a sheep, A. 109, Sh. 3921. It is clear that the material used for the third inoculation contained the virus at the time it was used, while it is almost certain that the virus was also present in the material used for the first and fourth inoculations though there were no control animals to prove this conclusively. The technique of the second operation cannot be considered, as yet, a proved method of obtaining infection. These results suggest that the sheep has a natural immunity to the virus, which even the actual introduction of the virus into the brain cannot break down.

A. 68, Sh. 3862—A. 86, Sh. 3879—A. 90, Sh. 3903. The first inoculation was on May 17th with material from a positive sheep, A. 65, Sh. 3855. The second inoculation was on June 7th with material from A. 75, Sh. 3876. A. 75, Sh. 3876, died four days after inoculation and histological examination of its brain and spinal cord was negative. The third inoculation was on June 27th with virus from A. 78, M. 3890, which "took" in the companion sheep A. 89, Sh. 3902; A. 91, Sh. 3904; and A. 92, Sh. 3905. As regards the first inoculation we know from A. 66, M. 3860, that the virus was present on the date of inoculation. We also know, as indicated above, that the virus was present in the material used for the third inoculation. This animal would seem, therefore, to have possessed a natural immunity to the disease.

A. 69, Sh. 3864—A. 94, Sh. 3907. The first inoculation was made on May 20th with material from A. 64, M. 3854. The second inoculation was made on June 27th and consisted of an emulsion of the frontal and occipital regions of the brain of A. 78, M. 3890. As regards the first inoculation we have no proof—by means of a control successfully-inoculated animal—that the virus was present in the material used, but inasmuch as this monkey presented the typical histological appearances of the disease, there is every reason to suppose that the virus was present. As regards the second inoculation, we have no proof that the virus is consistently present in the frontal and occipital areas of the brain, though histological examinations of affected animals would suggest that it is so, at least frequently. This sheep therefore also seems to have possessed a natural immunity.

A. 83, Sh. 3895—A. 114, Sh. 3933. The first inoculation was made on June 17th with material from A. 72, M. 3873, companion sheep, namely, A. 80, Sh. 3892 and A. 81, Sh. 3893, giving positive results. The second inoculation was made on July 29th from a positive sheep, A. 109, Sh. 3921. A companion animal of this last inoculation, which was also a previously inoculated animal, likewise remained unaffected, so that there was no control successful case for the second inoculation. The results of these two inoculations seem to show **again** a natural immunity in this animal.

A. 101, Sh. 3913—A. 125, Sh. 3948. The first inoculation was made with material which successfully conveyed the disease to A. 100, M. 3912; to A. 103, Sh. 3915; to A. 105, Sh. 3917; and probably to A. 102, Sh. 3914, which recovered. The virus was therefore evidently present in the material used on A. 101, Sh. 3913. The second inoculation was with material obtained from a positive sheep, A. 121, Sh. 3941. Decomposition was commencing in the carcase when the tissues were obtained for inoculation purposes. On the fourth and fifth days the inocu-

lated animal showed slight symptoms suggestive of encephalo-myelitis but recovered. Inoculations of the same material on the same day into another sheep, a calf, and a monkey (*Macacus cynomolgus*) were negative. The results of these experiments again suggest a natural immunity, not perhaps complete or varying from time to time in degree.

A. 104, Sh. 3916—A. 126, Sh. 3949. The same remarks apply to A. 104, Sh. 3916, regarding the first inoculation, as to the above-mentioned A. 101, Sh. 3913, the successful experiment in A. 103, Sh. 3915, being an exact counterpart. The second inoculation was also with the same material as was used on the above A. 125, Sh. 3948, but no illness resulted. Natural immunity to the virus, as evidenced by the first inoculation, seems to be again evident in this animal.

A. 119, Sh. 3939—A. 130, Sh. 3968. The first inoculation was with a Berkefeld filtrate of material which, unfiltered, conveyed the disease to A. 117, M. 3937, and to A. 121, Sh. 3941. After the second inoculation, with monkey material that successfully conveyed the disease to A. 129, M. 3967, an illness, probably encephalo-myelitis, developed, from which the sheep recovered. The inference drawn from these experiments is that the virus was not present, or was only present in subinfective amount, in the filtrate used.

A. 120, Sh. 3940—A. 131, Sh. 3969. These were parallel inoculations to those in the previous case. A possible mild attack of encephalo-myelitis followed the second inoculation. The same comment applies.

*Inferences drawn.* As regards the six sheep in which the final inoculations were successful, there seem to be valid reasons why the first were unsuccessful.

As regards the unsuccessful reinoculations, in the cases of the first three sheep the presence of a natural immunity to the introduction of the virus into the brain seems to be reasonably established. As regards the fourth sheep, there appears to be evidence of a natural immunity, perhaps not complete or varying in degree from time to time. The last two sheep suggest that the virus is held back, completely or to a large degree, by the pores of a Berkefeld filter.

#### APPENDIX XXVI. (a) *Calves showing Symptoms of Illness, possibly due to Encephalo-myelitis, after Intracerebral Inoculation of Material from the Brain and Spinal Cord of a Positive Monkey or a Positive Horse.*

A. 88. On June 27th this calf was inoculated intracerebrally with material from a monkey which successfully conveyed the disease on the same date to A. 87, M. 3900; A. 89, Sh. 3902; A. 91, Sh. 3904; A. 92, Sh. 3905; and A. 95, Horse 3908.

On July 1st the calf was sick and staggered, being especially weak in the hind legs. In the afternoon shivers were noticed in the hindquarters. On July 2nd it was very sick. It kept its head down, tended to walk in a circle, was very jerky on its legs, and stiff in the hindquarters and drowsy. In the evening it had convulsive movements, during which it bellowed, jumped in the air, fell down and struggled, and appeared to be unconscious. On July 3rd its hind legs swayed on movement, and it seemed weak and fell down at times. Next day it was better and could get up by itself from the ground, and was apparently almost normal in behaviour. On July 5th it could walk about, but swayed slightly on movement. On July 7th it was in much the same condition, though it had occasional convulsive seizures, and was not inclined to "play." It swayed a little on movement and there were occasional twitchings in one hind leg. On July 12th it was lying down and seemed drowsy. On July 14th it developed diarrhoea, which continued for about a fortnight, but from which it eventually recovered.

Considering the success of the material used in other animals, and the nature of the symptoms manifested, there seems little doubt that this calf had a mild form of encephalo-myelitis, from which it recovered.

A. 107. On July 10th this calf was inoculated intracerebrally with material from A. 95, the successfully inoculated horse. It seemed ill two days later, and on July 13th seemed very



sick. On July 14th it was weak and "wobbly" on the legs but otherwise normal. On July 16th it was better and thereafter showed no signs of illness until August 14th, when it received another inoculation (*vide infra* A. 127).

It is possible that the slight symptoms shown were due to a mild attack of encephalo-mylitis from which the animal recovered.

(b) *Calf showing no Symptoms after Intracerebral Inoculation (a second inoculation) of Brain and Spinal Cord from a Positive Sheep.*

A. 127. This calf is the same animal as has just been referred to under the designation A. 107. The second inoculation was made on August 14th and the calf showed no symptoms which could be attributed to encephalo-mylitis as the result of this inoculation.

It is possible that the first inoculation had rendered the animal immune to the later introduction of virus.

APPENDIX XXVII. *Failure to Convey the Disease to Dogs.*

Inoculation into A. 34, Dog 3806, failed on February 14th, while a monkey, A. 33, M. 3803, inoculated the day before with the same material developed the disease.

A. 16, Dog 3813, was inoculated on February 21st with material from A. 8, M. 3783, and failed to take, whilst A. 14, M. 3805, inoculated on February 13th with the same material developed the disease. During the eight days that elapsed between these inoculations, the virus may possibly have died out.

A. 56, Dog 3847, was inoculated from A. 50, M. 3839, unsuccessfully, whilst A. 55, M. 3848, inoculated on the same day with the same material developed the disease. This dog was the same animal as A. 16.

A. 97, Dog 3909. This was a third attempt to inoculate the animal shown as A. 16 and A. 56. This time fresh material from a positive sheep was used for the intracerebral inoculation. The result was again negative.

*Comment.* It is clear that in three of these inoculations, if not in all, the material used contained the virus. The dog has therefore not been shown to be susceptible to the disease.

APPENDIX XXVIII. *Failure to Convey the Disease to a Kitten by Intracerebral Inoculation.*

A. 79, Kitten 3891, received an intracerebral inoculation of an emulsion of brain and spinal cord from A. 72, M. 3873, and remained perfectly well afterwards, whereas A. 78, M. 3890, inoculated on the same day with the same material developed the disease.

APPENDIX XXIX. *Failure to Convey the Disease to Rabbits.*

A. 58, Rabbit 3849, was inoculated from A. 50, M. 3839, and failed to take. A. 55, M. 3848, inoculated with the same material, the day before, developed the disease.

A. 73, Rabbit 3874, inoculated from A. 66, M. 3860, failed to take, whilst A. 72, M. 3873, inoculated on the same day, developed the disease.

*Summary.* Two rabbits inoculated with material shown to contain the virus did not contract the disease.

APPENDIX XXX. *Doubtful Results in Guinea-pig Inoculations.*

Two guinea-pigs, A. 60, Gp. 3851, and A. 74, Gp. 3875, were inoculated. In the case of the first the material was obtained from a monkey the day after the same material conveyed the disease to A. 55, M. 3848, and A. 57, Calf 3848*b*. In the case of the second guinea-pig, the material used gave a positive result when inoculated on the same day into A. 72, M. 3873.

A. 60, Gp. 3851, six days after the inoculation was very sluggish and its hind legs seemed slightly weak, and on the following day it hardly moved at all. It died on the third day of illness. Histological examination showed some slight changes which might or might not be interpreted as evidence of a very mild form of encephalo-myelitis. The second guinea-pig, A. 74, Gp. 3875, remained apparently well until nearly a month after the inoculation, when its head was somewhat retracted and it could not raise itself up or walk. It died during the first day of this illness. Histological examination again showed some slight changes though these were probably not due to encephalo-myelitis.

*Summary.* One guinea-pig showed symptoms and histological lesions which might or might not be attributable to a very mild form of encephalo-myelitis. The second guinea-pig probably gave a negative result.

#### APPENDIX XXXI. *Failure to Convey the Disease to a Hen by Intracerebral Inoculation.*

A. 118, Hen 3938, received an intracerebral inoculation of material from A. 110, M. 3925, and remained perfectly unaffected whilst a monkey and a sheep inoculated on the same day with the same material developed the disease. This inoculation was specially made into a hen on account of the possibility of the human disease being the same as the spirochaetosis of fowls so common in the affected districts.

*Summary.* Virulent material failed to convey the disease to a hen by intracerebral inoculation.

#### APPENDIX XXXII. *Table showing the various Animal Inoculations.*

(Unless otherwise stated, the inoculations were made intracerebrally and the material comprised portions of the cortex of the cerebrum, pons, medulla, and spinal cord. + means a successful result, - means an unsuccessful result, and a blank indicates that the inoculated animal died too soon to allow manifestations of the disease to appear, supposing the virus had been transmitted. M. = monkey, Sh. = sheep, in. = inoculated, d. = died, k. = killed. Numbers in brackets [e.g. (A. 33)] mean that the animal so referred to received other inoculations under such designations. Roman figures represent generations.)

##### CASE 26, J. C. B., Narrabri.

(Ill four days; cerebro-spinal fluid taken 14/1/1918; swabbings of contacts taken 16/1/1918; patient died 15/1/1918.)

- A. 1 (A. 33), M. 3776 ( - ), cerebro-spinal fluid (in. 15/1/18).
- A. 2 (A. 13), M. 3777 ( - ), nasopharyngeal swabs, intraperitoneal (in. 17/1/18).
- A. 3 (A. 30, A. 44, A. 51), M. 3778 ( - ) nasopharyngeal swabs (in. 21/1/18).
- A. 4 (A. 22, A. 31), M. 3779 ( - ), nasopharyngeal swabs, into sciatic nerve (in. 22/1/18)

##### CASE 27, A. B., Narrabri.

- A. 5 (A. 21), M. 3780 ( - ), into sciatic nerve (in. 29/1/18).
- A. 6 (A. 20), M. 3781 ( - ), intraperitoneal (in. 29/1/18).
- A. 7, M. 3782.
- A. 8, M. 3783 ( + ), (in. 29/1/18; ill 7/2/18; k. 12/2/18).
- A. 9, M. 3785 ( + ), (in. 30/1/18; ill 11/2/18; k. 21/2/18).
- A. 10, M. 3786 ( + ), (in. 30/1/18; ill 14/2/18; k. 16/2/18).

*From A. 8, M. 3783, (in. 29/1/18; k. 12/2/18).*

- II. A. 11, M. 3801 ( - ), (in. 12/2/18; d. with miliary abscesses 25/2/18)
- A. 12, M. 3802 ( - ), Pasteur-Chamberland F. filtrate (in. 12/2/18).
- A. 13 (A. 2), M. 3804.
- A. 14, M. 3805 ( + ), (in. 13/2/18; ill 22/2/18; d. 25/2/18).



A. 15, Dog 3808.

A. 16, Dog 3813 ( - ), (in. 21/2/18).

A. 17 (A. 64), M. 3836 ( - ), material kept two months (in. 11/4/18).

*From* A. 9, M. 3785, (in. 30/1/18; k. 21/2/18).

A. 18 (A. 27), M. 3823 ( - ), (in. 15/3/18).

A. 19 (A. 26), M. 3824 ( - ), (in. 15/3/18).

*From* A. 11, M. 3801, (in. 12/2/18; d. from miliary abscesses 25/2/18).

A. 20 (A. 6), M. 3814 ( - ), (in. 27/2/18).

A. 21 (A. 5), M. 3815 ( - ), (in. 27/2/18).

A. 22 (A. 4, A. 31), M. 3818.

A. 23 (A. 52), Sh. 3816 ( - ), (in. 26/2/18).

#### CASE 28, J. M., Narrabri.

(Ill six days; died 6/2/18; cervical cord and adjacent part of brain only used.)

A. 24 (A. 29), M. 3798 ( - ), intraperitoneal (in. 9/2/18).

A. 25, M. 3799 (abscess in five days).

A. 26 (A. 19), M. 3807 ( - ), Pasteur-Chamberland F. filtrate (in. 15/2/18).

A. 27 (A. 18), M. 3809 ( - ), treated with serum (in. 16/2/18).

A. 28 (A. 43, A. 55 + ), M. 3810 ( - ), treated with serum (in. 16/2/18).

A. 29 (A. 24), M. 3819 ( - ), (in. 6/3/18).

#### CASE 36, J. K., Boggabri.

(Ill five days; died 8/2/18.)

A. 30 (A. 3, A. 44, A. 51), M. 3797 ( - ), nasopharyngeal swab (in. 9/2/18).

A. 31 (A. 4, A. 22), M. 3779 ( - ), filtrate of faeces (in. 9/2/18).

A. 32 (A. 41, A. 42), M. 3800 ( - ), cerebro spinal fluid (in. 11/2/18).

#### CASE 38, G. H., Wee Waa.

(Ill two days; died midnight 11-12/2/18.)

A. 33 (A. 1), M. 3803 ( + ), (in. 13/2/18; ill 25/2/18; k. 2/3/18).

A. 34, Dog 3806 ( - ), (in. 14/2/18).

A. 35 (A. 46, A. 65 + ), Sh. 3817 ( - ), (in. 26/2/18).

*From* A. 33, M. 3803 (k. 2/3/18).

II. A. 36 (A. 62 + ), M. 3834 ( - ), Noguchi culture (in. 4/4/18).

#### BROKEN HILL CASES.

A. 37 (A. 39, A. 66 + ), M. 3811 ( - ), case 6; patient ill five days; d. 13/2/18 (in. 21/2/18).

A. 38 (A. 47), M. 3812 ( - ), case 7; patient ill four days; d. 13/2/18 (in. 21/2/18).

A. 39 (A. 37, A. 66 + ), M. 3830 ( - ), case 18; patient ill eight days; d. 16/3/18 (in. 27/3/18).

#### BRISBANE CASE.

A. 40, M. 3828 ( - ), patient d. about ten days previously (in. 22/3/18).

#### NARRANDERA CASE.

A. 41 (A. 32, A. 42), M. 3831 ( - ), case 54, G.S.; ill twelve days; d. 22/2/18 (in. 27/2/18).

#### HORSE.

A. 42 (A. 32, A. 41), M. 3820 ( - ), horse d. 27/2/18 (in. 6/3/18).

#### FOWL TICKS, from Boggabri.

A. 43 (A. 28, A. 55 + ), M. 3827 ( - ), (in. 16/3/18).

*Acute Encephalo-myelitis*

## CASE 37, A. C. F. (J.), Boggabri.

(Ill three days; died 13/3/18.)

A. 44 (A. 3, A. 30, A. 51), M. 3825 ( - ), (in. 15/3/18).

A. 45 (A. 72 + ), M. 3837 ( - ), (in. 11/4/18).

A. 46 (A. 35, A. 65 + ), Sh. 3839 c ( - ), (in. 16/4/18).

## CASE 32, L. B., Narrabri.

(Ill five days; died 15/3/18.)

I. A. 47 (A. 38), M. 3826.

A. 48, M. 3829 ( + ), (in. 22/3/18; ill 2/4/18; k. 4/4/18).

*From A. 48, M. 3829 (k. 4/4/18).*

II. A. 49, M. 3835 ( + ), (in. 4/4/18; ill 11/4/18; k. 15/4/18).

*From A. 49, M. 3835 (k. 15/4/18).*

III. A. 50, M. 3839 ( + ), (in. 15/4/18; ill 20/4/18; k. 24/4/18).

A. 51 (A. 3, A. 30, A. 44), M. 3840.

A. 52 (A. 23), Sh. 3839 b ( + ), (in. 16/4/18; ill 19/4/18; d. 22/4/18).

A. 53 (A. 91 + ), Sh. 3863 ( - ), (in. 17/5/18).

*From A. 50, M. 3839 (k. 24/4/18).*

IV. A. 54 (A. 71), M. 3846 ( - ), Pasteur-Chamberland F. filtrate (in. 24/4/18).

A. 55 (A. 28, A. 43), M. 3848 ( + ), (in. 25/4/18; ill 3/5/18; d. 5/5/18).

A. 56 (A. 16, A. 97), Dog 3847 ( - ), (in. 25/4/18).

A. 57, Calf 3848 b ( + ), (in. 25/4/18; ill 30/4/18; d. 2/5/18).

A. 58, Rabbit 3849 ( - ), (in. 26/4/18).

A. 59, Rabbit 3850.

A. 60, G. pig 3851 (?), (in. 26/4/18; ill 2/5/18; d. 4/5/18).

A. 61, Kitten 3852.

*From A. 52, Sh. 3839 b (d. 22/4/18).*

A. 62 (A. 36), M. 3845 ( + ), (in. 23/4/18; ill 29/4/18; k. 3/5/18).

*From A. 62, M. 3845 (k. 3/5/18).*

V. A. 63 (A. 76, A. 93, A. 115), Sh. 3853 ( - ), (in. 3/5/18).

*From A. 55, M. 3848 (d. 5/5/18).*

A. 64, M. 3854 ( + ), (in. 7/5/18; ill 14/5/18; k. 18/5/18).

A. 65 (A. 35, A. 46), Sh. 3855 ( + ), (in. 7/5/18; ill 13/5/18; d. 14-15/5/18).

*From A. 65, Sh. 3855 (d. 14-15/5/18).*

VI. A. 66 (A. 37, A. 39), M. 3860 ( + ), (in. 17/5/18; ill 22/5/18; k. 30/5/18).

A. 67 (A. 77, A. 89), Sh. 3861 ( - ), intraperitoneal (in. 17/5/18).

A. 68 (A. 86, A. 90), Sh. 3862 ( - ), (in. 17/5/18).

*From A. 64, M. 3854 (k. 18/5/18).*

A. 69 (A. 94), Sh. 3864 ( - ), (in. 20/5/18).

A. 70 (A. 92 + ), Sh. 3865 ( - ), nose swabbed (in. 20/5/18).

A. 71 (A. 54), M. 3872 ( - ), lumbar puncture, venous injection (in. 27/5/18).

*From A. 66, M. 3860 (k. 30/5/18).*

VII. A. 72 (A. 45), M. 3873 ( + ), (in. 30/5/18; ill 13/6/18; k. 15/6/18).

A. 73, Rabbit 3874 ( - ), (in. 30/5/18).

A. 74, G. pig 3875 (?), (in. 30/5/18; ill 25/6/18; d. 25/6/18).

A. 75, Sh. 3876 ( - ), d. in four days (in. 31/5/18; d. 4/6/18).

A. 76 (A. 63, A. 93, A. 115), Sh. 3877 ( - ), lumbar puncture, venous injection (in. 31/5/18).

A. 77 (A. 67, A. 89), Sh. 3878 ( - ), dried brain injected (in. 7/6/18).

*From A. 72, M. 3873 (k. 15/6/18).*

VIII. A. 78, M. 3890 ( + ), (in. 15/6/18; ill 25/6/18; k. 27/6/18).

A. 79, Kitten 3891 ( - ), (in. 15/6/18).



- A. 80, Sh. 3892 ( + ), (in. 17/6/18; ill 24/6/18; k. 28/6/18).  
 A. 81, Sh. 3893 ( + ), (in. 17/6/18; ill 29/6/18; k. 2/7/18).  
 A. 82, Sh. 3894 ( - , ? recovery), (in. 17/6/18; ill ? 29/6/18; d. 10/7/18).  
 A. 83 (A. 114), Sh. 3895 ( - ), (in. 17/6/18).  
 A. 84 (A. 109), Sh. 3896 ( - ), frontal and occipital only (in. 17/6/18).  
 A. 85 Sh. 3897 (death in 2 days), medulla only.

*From A. 75, Sh. 3876 (sheep histologically negative, d. 4/6/18).*

- A. 86 (A. 68, A. 90), Sh. 3879 ( - ), (in. 7/6/18).

*From A. 78, M. 3890 (k. 27/6/18).*

- IX.** A. 87, M. 3900 ( + ), (in. 27/6/18; ill 4/7/18; k. 7/7/18).  
 A. 88, Calf 3901 (recovered ?), (in. 27/6/18; ill 2-7/7/18).  
 A. 89 (A. 67, A. 77), Sh. 3902 ( + , recovering ?), (in. 27/6/18; ill 2-4/7/18; d. 4/8/18).  
 A. 90 (A. 68, A. 86), Sh. 3903 (recovered ?), (in. 27/6/18; ill 2 and 3/7/18).  
 A. 91 (A. 53), Sh. 3904 ( + ), (in. 27/6/18; ill 3/7/18; d. 3/7/18).  
 A. 92 (A. 70), Sh. 3905 ( + ), medulla only (in. 27/6/18; ill 3/7/18; d. 6/7/18).  
 A. 93 (A. 63, A. 76, A. 115), Sh. 3906 ( - ?), medulla only (in. 27/6/18; ill? 2/7/18).  
 A. 94 (A. 69), Sh. 3907 ( - ), frontal and occipital only (in. 27/6/18).  
 A. 95, Horse 3908 ( + ), (in. 28/6/18; ill 7/7/18; k. 9/7/18).  
 A. 96, Sh. 3935 ( - ), (in. 29/7/18).

*From A. 81, Sh. 3893 (k. 2/7/18).*

- A. 97 (A. 16, A. 56), Dog 3909 ( - ), (in. 2/7/18).

*From A. 92, Sh. 3905 (d. 6/7/18).*

- X.** A. 98, Sh. 3910 ( + ), (in. 6/7/18; ill 11/7/18; d. 15/7/18).  
 A. 99, Sh. 3911 (death in 2 days).

*From A. 87, M. 3900 (k. 7/7/18).*

- A. 100, M. 3912 ( + ), (in. 7/7/18; ill 16/7/18; k. 17/7/18).  
 A. 101 (A. 125), Sh. 3913 ( - - ), (in. 7/7/18).  
 A. 102, Sh. 3914 (recovered ?), (in. 7/7/18; ill 12-16/7/18).  
 A. 103, Sh. 3915 ( + ), emulsion plus serum of A. 82, Sh. 3894 (in. 7/7/18; ill 14/7/18; k. 17/7/18).  
 A. 104 (A. 126), Sh. 3916 ( - ), emulsion plus serum of A. 82, Sh. 3894 (in. 7/7/18).  
 A. 105, Sh. 3917 ( + ), emulsion plus normal sheep's serum (in. 7/7/18; ill 14/7/18; d. 16-17/7/18).  
 A. 106, Sh. 3918 ( - , death in 5 to 6 days), emulsion plus normal sheep's serum (in. 7/7/18; d. 12/7/18.)

*From A. 95, Horse 3908 (k. 9/7/18).*

- A. 107 (A. 127), Calf 3919 (recovered ?), (in. 10/7/18; ill on 13 and 14/7/18).

*From A. 98, Sh. 3910 (d. 15/7/18).*

- XI.** A. 108, Sh. 3920 ( + ), (in. 16/7/18; ill 22/7/18; d. 23/7/18).

*From A. 103, Sh. 3915 (k. 17/7/18).*

- A. 109 (A. 84), Sh. 3921 ( + ), (in. 18/7/18; ill 25/7/18; d. 27/7/18).

*From A. 100, M. 3912 (k. 17/7/18).*

- A. 110, M. 3925 ( + ), (in. 19/7/18; ill 30/7/18; k. 2/8/18, might have recovered).  
 A. 111, Sh. 3922 ( - ), emulsion plus serum of A. 89, Sh. 3902 (in. 19/7/18).  
 A. 112, Sh. 3925 (d. in four days), emulsion plus serum of A. 89, Sh. 3902.  
 A. 113, Sh. 3924 (d. in two days), emulsion plus serum of A. 89, Sh. 3902.

*From A. 109, Sh. 3921 (d. 27/7/18).*

- XII.** A. 114 (A. 83), Sh. 3933 ( - ), (in. 29/7/18).  
 A. 115 (A. 63, A. 76, A. 93), Sh. 3934 ( - ), (in. 29/7/18).

*From A. 110, M. 3925 (k. 2/8/18).*

- A. 116 (A. 132), M. 3936 ( - ), emulsion plus serum of A. 89, Sh. 3902 (in. 2/8/18).  
 A. 117, M. 3937 ( + ), emulsion plus serum of A. 102, Sh. 3914 (in. 2/8/18; ill 25/8/18; k. 27/8/18).

- A. 118, Hen 3938 ( - ), (in. 2/8/18).  
A. 119 (A. 130), Sh. 3939 ( - ), Berkefeld filtrate (in. 2/8/18).  
A. 120 (A. 131), Sh. 3940 ( - ), Berkefeld filtrate (in. 2/8/18).  
A. 121, Sh. 3941 ( + ), (in. 2/8/18; ill 8/8/18; d. 13/8/18).  
A. 122, Sh. 3942 ( - ), (in. 2/8/18).  
A. 123, Sh. 3943.  
A. 124, M. 3952 ( + ), (in. 17/8/18; ill 3/9/18; k. 7/9/18).

*From* A. 121, Sh. 3941 (d. 13/8/18).

- XIII.** A. 125 (A. 101), Sh. 3948 (recovered ?), (in. 14/8/18; ill on 18 and 19/8/18).  
A. 126 (A. 104), Sh. 3949 ( - ), (in. 14/8/18).  
A. 127 (A. 107), Calf 3950 ( - ), (in. 14/8/18).  
A. 128, M. 3951.

*From* A. 117, M. 3937 (k. 27/8/18).

- A. 129, M. 3967 ( + ), (in. 27/8/18; ill 6/9/18; k. 9/9/18).  
A. 130 (A. 119), Sh. 3968 (recovered?), (in. 28/8/18; ill 5-10/9/18).  
A. 131 (A. 120), Sh. 3969 ( - ), (in. 28/8/18).

*From* A. 129, M. 3967 (k. 9/9/18).

- XIV.** A. 132 (A. 116), M. 3977 ( - ), (in. 11/9/18).



## ON THE EFFECTS OF INJECTIONS OF QUININE INTO THE TISSUES OF MAN AND ANIMALS.

BY LEONARD S. DUDGEON, C.M.G., C.B.E., F.R.C.P. (LOND.),

Colonel A.M.S., Consulting Bacteriologist B.S.F., and Member of the  
Advisory Committee to the War Office for Infectious Diseases  
and Sanitation, Eastern Mediterranean.

(With Plate III.)

IN December 1917, Lieut.-Col. MacGilchrist of the Indian Medical Service published a paper on the necrosis produced by intra-muscular injections of strong solutions of quinine salts<sup>1</sup>. It might be an advantage to quote the first few lines of this communication: "Advocates of intra-muscular injections of strong solutions of quinine salts for the treatment of malaria seldom omit to state that no local ill-effects are produced." He records a case of tissue necrosis following on the intra-muscular injection of eleven grains of quinine bi-hydrochloride in thirty-four minims of water. Death supervened thirteen hours later. MacGilchrist especially noted, owing to rapid tissue necrosis, that the track of the needle remained patent. He regards as an established fact that most of the quinine injected is precipitated and probably chemically combined with serum proteins in the necrosed tissues and for this reason intra-muscular injections of concentrated solutions of quinine salts are not to be recommended for cases of emergency. Very dilute solutions of quinine salts are, in his opinion, rapidly and completely absorbed whether employed subcutaneously or by the intra-muscular route. If the views which MacGilchrist puts forward in this and other communications are to be accepted without reserve then intra-muscular injections of strong solutions of quinine should no longer be employed. It was for this reason that Major-General Sir M. P. C. Holt, K.C.B., K.C.M.G., D.M.S., B.S.F., asked me to carry out an experimental enquiry on animals as to the effects produced by intra-muscular injections of strong solutions of quinine.

Human muscle was examined from fatal cases of malaria or suspected malaria which had received an injection of quinine at periods varying from one hour to three months from the time of the inoculation, and in some instances an estimation of quinine in the affected tissues was made. For the experimental enquiry, cast mules, rabbits, guinea-pigs, and frogs were used, while the preparations of quinine employed were (a) bi-hydrochloride in saline,

<sup>1</sup> *Indian Med. Gaz.* LII. No. 12.

(b) acid sulphate in saline<sup>1</sup>, (c) quinine alkaloid dissolved in alcohol (about the strength of hospital brandy), (d) and in ether (medicinal). The alkaloid shaken in blood serum and saline—a preparation which consisted of one gramme of alkaloid, one c.c. of 90 per cent. alcohol, and olive oil to 3 c.c.—and a somewhat similar preparation to the last mentioned was tested directly after it arrived in the East for trial. The quinine solutions have been injected in concentrated and dilute solutions. The preparations most commonly employed for intra-muscular injections were the bi-hydrochloride of quinine in saline, and, to a less extent, bi-hydrochloride dissolved in brandy. Human muscle was obtained in all instances from cases which had received quinine in some form in concentrated solutions as commonly employed for the treatment of malaria. Control observations were made on the action on the tissues of animals of acids and ether (quinine solvents).

Numerous cases were treated with intra-muscular injections of quinine bi-hydrochloride dissolved in brandy on the basis of three grains of the salt per 10 lb. of body weight.

Experiments have also been made on the absorption of quinine from the seat of inoculation, at periods varying from a few minutes to several weeks, and as to the question of the storage of the alkaloid in the heart-muscle, liver and kidneys.

All who have had experience of malarial patients are aware that an apparent anaemia occurs, and a true anaemia with considerable blood destruction. Further, a severe haemolytic anaemia and haemoglobinuria is associated with malaria and may occur at a period of the disease when quinine treatment is essential. For these reasons it was necessary to induce anaemia and haemoglobinuria in animals by means of immune sera so as to observe whether intra-muscular injections of quinine in concentrated and dilute solutions excited a more intense tissue reaction than in the control animals.

All the chemical estimations of residual quinine were undertaken by Captain C. E. C. Ferrey, O.B.E., R.A.M.C. (T.F.), Analytical Chemist to the Central Laboratory, B.S.F., who employed the Stas-Otto process for these investigations.

#### QUININE AND HAEMOLYSIS.

Although the purpose of these experiments was to observe the effects of intra-muscular injections of quinine on the tissues, yet, attention must be drawn to the haemolytic activity of quinine as estimated *in vitro*. Two solutions were prepared for the purpose: (1) 1 per cent. bi-hydrochloride of quinine in saline, (2) a solution of hydrochloric acid in saline of the same total acidity as the quinine solution—0.18 per cent. The total bulk of test solution and saline in each tube was 1 c.c. The haemolytic end point of the quinine solution, acting at 37° C. for five hours, was 0.16 c.c., while the acid solution alone induced haemolysis down to 0.1 c.c. Normal saline was employed throughout

<sup>1</sup> Only three experiments were made with this salt as it was found to excite more intense necrosis than the other preparations which were employed.



the experiments as the diluting agent. Further, the haemolytic action was much more rapid with the acid solution than with the bi-hydrochloride of quinine in saline. Normal human serum or citrated plasma in suitable amounts prevented haemolysis. These results briefly referred to serve to illustrate the haemolytic action of bi-hydrochloride of quinine and of the corresponding acid. It will be readily appreciated that the preparations of quinine employed for intra-muscular injections in the treatment of malaria are of considerably greater strength than in these experiments on haemolysis *in vitro*. The haemolytic activity of a 1 per cent. solution of bi-hydrochloride of quinine is extremely rapid, while strong solutions produce instantaneous haemolysis. The alkaloid when suspended in saline induces a much more gradual haemolysis than the salt in solution, although for obvious reasons a considerable error must occur in estimating the haemolytic action of varying suspensions of quinine alkaloid in saline. It has been found that the agglutination of human or other red cells, well known to be induced by free acids, is absent in the standard acid solutions of bi-hydrochloride of quinine employed in suitable strengths for such purposes. If 0.05 c.c. of the standard solution of hydrochloric acid already referred to is made up to a total volume of 1 c.c. an immediate agglutination occurs on the addition of human red cells, while with 0.15 c.c. of the 1 per cent. solution of bi-hydrochloride of quinine in 1 c.c. saline no such effect is induced. All experiments tend to show that although quinine acts as a powerful haemolytic agent the acid employed to dissolve the alkaloid is much more potent in its effect.

#### WHAT IS THE COMPARATIVE EFFECT OF INTRA-MUSCULAR INJECTIONS OF CONCENTRATED AND DILUTE SOLUTIONS OF QUININE?

Numerous experiments have been made to solve this question concerning which information was specially required.

The following detailed descriptions express the essential features:

(1) A rabbit received an intra-muscular injection of 0.039 gramme of bi-hydrochloride of quinine in 1 c.c. saline, and the same quantity of quinine in 3 c.c. of saline into another set of muscles.

The following day 0.078 gramme of the same preparation in 2 c.c. of saline was injected in the right side and a similar quantity in 4 c.c. of saline in the left.

The post-mortem examination took place on the following day. The results showed that no advantage was gained by injecting the quinine in twice the quantity of saline. The spreading oedema was greater owing to the increase in bulk of fluid injected, while the inflammatory process and muscle necrosis in both instances was so evident that no importance could be attached to minute differences in the affected tissues. If quinine injections are given in such dilutions that the action of quinine and free acids on the red cells and tissues is reduced to a minimum, then the bulk of fluid required would nullify, in my opinion, any advantage that might be gained. Further there is a greater

possibility of suppuration from injection of quinine in large bulk into the tissues at frequent intervals.

(2) Four rabbits were injected by the intra-muscular route as follows:

- A. 0.018 gramme of bi-hydrochloride of quinine in 0.5 c.c. saline (concentrated).
- B. 0.018 gramme of bi-hydrochloride of quinine in 0.5 c.c. normal rabbit serum (concentrated).
- C. 0.018 gramme of bi-hydrochloride of quinine made up to 2 c.c. with auto-rabbit serum.
- D. 0.7 c.c. of 1:5000 solution of quinine alkaloid in ether.
- E. 0.18 gramme of bi-hydrochloride of quinine made up to 2 c.c. with auto-rabbit serum and injected by the intra-muscular route.

In every instance obvious necrosis occurred. The interval between the time of the injection and the autopsy was six days in the case of experiments A and B, 4 days in C and E, and 3 days in D.

The most intense changes were induced when the alkaloid was injected with ether. Necrosis of muscles in all stages—wide areas of polynuclear and mononuclear inflammation especially towards the capsular area—thrombosis of vessels—red cell agglutination and haemolytic changes in the tissue blood as well as in the intra-vascular blood.

(3) A mule was injected with 1 gramme of alkaloid of quinine in alcohol and olive oil (3 c.c.). On the following day with 1 gramme of the alkaloid in 10 c.c. ether.

Although the total bulk of fluid varied considerably in each inoculation the final results were similar; further, the concentration of quinine in the solutions employed did not affect the final results. In each instance extensive necrosis—haemorrhage into the tissues—congestion of blood vessels—and foci of acute inflammation were met with. Numerous experiments have been referred to elsewhere in this communication which serve to illustrate the comparative effects of concentrated and dilute solutions when injected into the tissues of animals.

#### THE MICROSCOPICAL FINDINGS AS A RESULT OF INTRA-MUSCULAR INJECTIONS OF QUININE SALTS AND ALKALOID QUININE.

The first effect of an injection of a quinine preparation into the tissues is necrosis of muscle. The fibres most affected are completely necrosed leaving the empty sheaths. Oedema of the tissues accompanies the necrosis, together with agglutination of red cells and haemolysis in the vessels and in the blood which has escaped into the tissues. These changes are in evidence within ten minutes of the inoculation. At this period no leucocytic reaction has occurred, but an intense congestion of all vessels in the area has taken place. At the end of one hour the leucocytic reaction may not be a marked feature, although in preparations of quinine which include oil an intense polynuclear leucocytic reaction has been observed. The muscle fibres in the necrotic areas, as time advances, present appearances which are various as well as distinctive. In addition to the fibres which are completely necrosed others show fragmentation so that numerous “apparent droplets” are observed within the sheaths (Pl. III,



Fig. 1). Owing to a multiplication of the nuclei of the sheaths which occurs and a collapse of the sheaths themselves, the fibrous framework of the new tissue is gradually formed. Shrunken fibres with several nuclei give the well-known appearance of pseudo-giant cell formation. The large tissue cells act as phagocytes for fat droplets, red cells and free iron granules. Active destruction of the vessel walls occurs together with thrombosis which may or may not serve as a conservative process (Pl. III, Fig. 3). Polynuclear inflammation of the necrosed walls can be clearly demonstrated. Active changes occur in the vessels apart from thrombosis such as agglutination of red cells and haemolytic phenomena while similar changes are met with outside the vessel walls. In certain instances marked red cell agglutination occurs within the vessel without the evidence of inflammation (Pl. III, Fig. 2).

In course of time absorption of muscle fragments and of inflammatory products takes place so that finally we have a condition of fibrous myositis such as is met with as a result of traumatic influence or syphilis. Nerve fibres are implicated in the earliest stages. It is not uncommon to observe that haemorrhages and large vacuoles occupy the space of the original nerve fibres. Fibrous tissue formation surrounds the nerves in the muscles and also the individual fibres. Large nerve trunks may be so implicated by the oedema and spreading necrosis that complete nerve degeneration occurs.

Strands of necrosed muscle fibres may persist for ten to fourteen days after an injection of quinine, possibly owing to the diminished tissue absorption which must occur as a result of quinine inoculation. The bulk of quinine however is absorbed from the tissues with extreme rapidity—simply a question of hours.

#### EXPERIMENTS TO ILLUSTRATE THE EFFECTS ON THE TISSUES OF CERTAIN PREPARATIONS OF QUININE.

A cast mule was injected with the following preparations. The results were as follows:

Number of inoculation	Site of inoculation	Nature of inoculation	Naked eye appearances at autopsy	Interval between inoculation and examination
1	Back, near side	10 c.c. ether	Extensive necrosis, 6 or 7 inches long, 3-4 inches wide. Slight superficial oedema	6 days
2	Near fore leg	ditto	Extensive tissue necrosis. Slight oedema	5 days
3	Back, off side	0.25 gm. of alkaloidal quinine in 10 c.c. ether	Widespread oedema. Very extensive necrosis	4 days
4	Off fore leg	ditto	ditto	3 days
5	Near hind leg	5 c.c. of 4.3 % HCl in saline	Extensive oedema spreading along muscle septa. Marked necrosis	2 days
6	Off hind leg	5 c.c. of 25% bi-hydrochloride of quinine in saline (same total acidity as above (5))	ditto	24 hours

This experiment gives an excellent survey of the effect produced by ether-quinine alkaloid dissolved in ether—bi-hydrochloride of quinine in saline—hydrochloric acid made to the same total acidity as the solution of quinine. In Experiment 3 of this series 0.25 gramme of alkaloidal quinine was injected in no less than 10 c.c. ether, yet the effect on the tissues was considerable. Here the quinine was given in dilute solution, but the solvent employed was largely responsible for the effect. Experiments 5 and 6 of this series show similar effects on the tissues, yet quinine was not employed in Series 5. The result must have been due therefore to the solvent. This fact must never be lost sight of in any discussion on the question of tissue necrosis induced by quinine, as will be referred to elsewhere in this communication.

#### INTRA-MUSCULAR INJECTIONS OF ALKALOIDAL QUININE IN 60 PER CENT. ALCOHOL.

Several animals received intra-muscular injections of quinine dissolved in alcohol, either concentrated or in dilute solutions. The rabbit used in the experiment to be referred to in detail was injected daily for six days with 0.04 gramme in 1 c.c. of 60 per cent. alcohol. Blood counts were made with the object of determining whether a leucocytosis would be induced or an alteration in the red cell-haemoglobin system. The injections were made in a different muscular area on each occasion. Certain tissues were preserved at the autopsy for quinine estimation, and the entire local area was removed for a similar purpose except for a small portion reserved for microscopy. First injection: there was marked oedema and a long line of muscle necrosis. A typical fibromyositis had been produced around the necrosed muscle in which all possible changes were recognised. Numerous new formed blood vessels were present, also blood pigment and scattered haemorrhages. Degeneration of the vessel walls was evident. The effect of the second inoculation was similar except that a well-developed interstitial neuritis was found in the nerve from the affected area. No quinine was detected in the local lesion. The third injection had produced a large haemorrhagic area superficial to a diffuse black-brown necrosis of muscle. Numerous foci of polynuclear cells were present. No quinine was obtained from the local lesion. Fourth injection made four days before death showed very considerable oedema of muscle and necrosis. Thromboses of some of the large vessels were present with necrosis of the muscular walls. The degenerated muscle fibres were fragmented and were undergoing a process of gradual absorption by which highly cellular patent sheaths remained, the walls of which ultimately coalesced and thus assisted in the formation of the fibrous scar.

Fifth injection. Diffuse haemorrhage and oedema together with a prominent area of greyish brown, dry, muscular necrosis had occurred.

Sixth injection. This was completed forty-eight hours before death. The changes noted at the autopsy were similar to those referred to as a result



of the previous inoculation. The microscopical appearances were similar, but of a more acute type. There was a very marked polynuclear inflammation, abundant haemorrhages, and active haemolytic changes. The muscle fibres were necrosed, fragmented, and broken up into coarse granules, and haemorrhages had occurred among the nerve bundles. Both kidneys and liver removed at the autopsy forty-eight hours after the last intra-muscular injection were free from quinine, although 0.24 gramme of alkaloidal quinine had been injected in eight days, and the last injection was only forty-eight hours before death. It will be readily appreciated from those experiments described in detail that alkaloidal quinine given in 60 per cent. alcohol excites intense changes in the tissues in the inoculated area.

*Intra-muscular Injections of Quinine Alkaloid in Alcohol*

Differential count of leucocytes. 1st column=%, 2nd column=no. per c.mm.													
Dates	Weights	Red cells per c.mm.	H.B.	C. I. (Colour- Index)	Leuco- cytes per c.mm.	Nucleated red cells, No. to % leucocytes	Finely granular oxyphil		Lympho- cytes		Large hyalines		Quinine alkaloid in 60% alcohol. Intra-muscular injections. Total bulk of fluid=1 c.c.
							1	2	1	2	1	2	
15/7/18	1000 grms.	5,400,000	83 %	0.7	8980	2	43.0	3827	55.0	4895	1.5	133	0.04 gramme
16/7/18	950 „	—	—	—	7900	1	48.5	3831	50.0	3950	1.0	79	0.04 „
17/7/18	950 „	5,200,000	84 %	0.8	7600	3	27.5	2190	38.0	5168	3.5	266	0.04 „
18/7/18	955 „	—	—	—	7020	1	32.5	2275	61.0	4270	5.5	385	0.04 „
19/7/18	1000 „	5,200,000	82 %	0.7	7100	3	27.5	1952	65.0	4615	6.5	461	0.04 „
20/7/18	1000 „	5,300,000	82 %	0.7	7150	—	—	—	—	—	—	—	0.04 „
21/7/18	950 „	5,000,000	80 %	0.8	6990	1	27.0	1863	63.0	4347	10.0	690	
22/7/18	923 „	5,200,000	84 %	0.8	7520	0	22.0	1650	68.0	5100	8.0	600	Rabbit killed

THE INJECTION OF QUININE ALKALOID WITH OLIVE OIL.

The following preparation was tried for intra-muscular injections:

Quinine alkaloid	...	...	1 gramme.
Alcohol 90 per cent.	...	...	1 c.c.
Olive oil (neutral)	...	...	to 3 c.c.

Injections were made of 0.2 c.c. of the above mixture into rabbits. Some animals received one injection, others as many as five on successive days. The chief features in such experiments are concentration of the alkaloid and the introduction of a fatty substance on the assumption that it would act as a tissue protector. To avoid needless repetition it will be sufficient to refer to one experiment in detail.

A rabbit received five intra-muscular injections of the alkaloid in oil commencing with 1/10 c.c. containing half a grain of quinine on five successive days. Each inoculation was made into a different area of fresh tissue. The animal was killed eleven days from the first commencement of the experiment. During the period it lost 160 grammes in weight. No advantage would be gained by recording the tissue changes met with in each muscular area. The

naked eye appearances were profound in each focus as occurs with all concentrated injections of quinine, while the oil exerted no beneficial action. On the other hand an injection of one grain of the alkaloid for a rabbit must be regarded as a full dose. The muscles were soft, showed large areas of necrosis, thrombosis of vessels and haemorrhages into the muscular tissue. The red haemorrhagic zone was sharply defined from the necrosed pale area.

Microscopically an extensive fibro-myositis had developed with diffuse muscle necrosis. A polynuclear inflammation was far more in evidence than has occurred in experiments with other preparations of quinine. Nerve fibres embedded in the muscle tissue were degenerated, while in the older lesions fibrous tissue could be recognised among the fibres and surrounding them.

The second injection amounted to 1 grain of the alkaloid in a total bulk of 1/5 c.c. It had the advantage, therefore, for this experiment of concentration. The inoculation was made in the muscles in the front of the thigh. At the autopsy 10 days later it was found that the necrosis had spread to the muscles at the back of the thigh and the great sciatic nerve was surrounded by necrosed and inflammatory tissue. The nerve was removed entire and sections were prepared in "Marchi" at various levels. An extreme degree of nerve degeneration was noted, in fact, very few of the fibres escaped (Pl. III, Fig. 4). Some were completely degenerated, others patchy, while actual haemorrhages into the nerve had taken place. Here we have an example of the destruction of the sciatic nerve following an injection of quinine. The inoculation was made distant from the nerve, directly into a muscle, and in such a manner that escape of fluid was well-nigh impossible. The necrosis, however, was so excessive that it had extended right through the thigh muscles and implicated the sciatic nerve with disastrous results for the animal. This fact, however, will be referred to later. The entire liver and both kidneys were examined for evidence of quinine storage, but no alkaloid was detected.

#### THE INJECTION OF QUININE ALKALOID IN CREOSOTE AND FAT.

A preparation which was sent to Macedonia for trial consisting of one gramme of quinine alkaloid, 0.75 c.c. of beechwood creosote and 5 c.c. of neutral fat was not found to possess any special advantage over the other preparations referred to. Mules received intra-muscular injections of the above preparation causing extensive necrosis, and a gelatinous spreading subcutaneous oedema. A lesion some 6½ inches long, 3 inches deep, and 2½ inches wide was produced by the injection of 10 c.c. into the muscles of a mule. Chemical analyses of the necrosed muscle showed that the quinine had been absorbed with rapidity. Two experiments will be quoted to illustrate this point.

	Amount injected	Date	Post-mortem date	Amount of quinine recovered
Experiment 1.	5 c.c.	21st	25th	0.003 gramme
„ 2.	5 c.c.	22nd	25th	0.063 „

Similar evidence in the case of human muscle was also obtained.



*A cast brown horse was inoculated intra-muscularly with bi-hydrochloride of quinine in saline as illustrated in the accompanying table:*

No. of experiment	Amount of bi-hydrochloride of quinine employed	Interval between inoculation and autopsy	Naked eye appearance of the tissues at the autopsy
1	2.5 grms. in 5 c.c. saline	13 days	Marked necrosis and fibrosis.
2	1.125 grms. in 2.5 c.c. saline	8 days	Localised necrosis separated from a fibrous zone by a line of haemorrhage
3	4.5 grms. in 10 c.c. saline	3 days	Long tract of necrosis with marked oedema of muscle
4	2.25 grms. in 5 c.c. saline	2 days	Localised necrosis with oedema
5	1.125 grms. in 2.5 c.c. saline	30 hours	Very extensive gelatinous oedema with localised necrosis
6	1.125 grms. in 2.5 c.c. saline	1½ hours	Localised necrosis with wide tracts of haemorrhage

A brief account of the microscopical appearances of the lesions referred to in the previous table will be recorded.

No. 1. Fibrous myositis with complete capsulation of the necrotic muscular tissues. Active polynuclear inflammation present.

No. 2. Fibrous myositis completely enclosing necrosed muscle—haemorrhagic foci and polynuclear inflammation.

No. 3. Extensive necrosis of muscular tissue and of the walls of blood vessels resulting in haemorrhage and active haemolytic changes in the diffused blood. Agglutination of red cells very obvious.

No. 5. Wide necrosis of muscular tissue and an intense leucocytic reaction in and around muscle fibres which are fissured, fragmented, and show all stages of degeneration. Active haemolytic changes and masses of agglutinated red cells present.

No. 6. Diffuse muscle necrosis with long tracts of oedematous tissue evident. Haemorrhagic foci and active haemolytic phenomena present. Walls of blood vessels necrosed and ruptured.

*A cast grey mule was injected intra-muscularly with certain solutions of quinine with the following results:*

No. of experiment	Amount of quinine injected and total bulk of fluid	Amount obtained at autopsy	Interval between the intra-muscular injections and the P.M. examination	Appearances of the muscle lesions at the autopsy
1	4.1 grms. alkaloid in 10 c.c. of 70 % alcohol	0.014 gramme	8 days	Fibrosis and extensive necrosis
2	4.5 grms. bi-hydrochloride in 10 c.c. saline	0.020 „	7 „	Central black necrosis, scattered haemorrhages. Gelatinous oedema
3	2.25 grms. bi-hydrochloride in 5 c.c. saline	0.003 „	6 „	Central necrosis with haemorrhagic zone at periphery. Gelatinous oedema
4	Ditto	0.0085* „	5 „	Ditto (Pl. III, Fig. 5)
5	1.125 grms. bi-hydrochloride in 2.5 c.c. saline	0.012 „	4 „	Changes similar, but lesions less extensive
6	4.1 grms. alkaloid in 10 c.c. of 70 % alcohol	0.074 „	3 „	Extensive gelatinous oedema surrounding a wide area of dry necrosis (7)
7	2.05 grms. alkaloid in 5 c.c. of 70 % alcohol	0.048 „	2 „	Central black necrosis with oedematous tissue around
8	2.25 grms. bi-hydrochloride in 5 c.c. saline	0.938 „	1 hour	Extensive dry necrosis about 4 inches square

\* This does not represent the sum total because some of the extract was blown out of a flask.

In each instance a different area of the animal's body was used for the injection.

The mule was killed one hour after the last inoculation. At the post-mortem examination, the affected tissues in the region of the injections and some of the surrounding healthy tissue were removed for estimation of quinine content, while a small portion was reserved for microscopy.

We can infer from the results of the chemical investigation of the muscle lesions as illustrated in the accompanying table that the bulk of the quinine is absorbed whether injected as an alkaloid in alcohol, or as the bi-hydrochloride in saline. It was also found that at the expiration of one hour from the time of the inoculation to the death of the animal only 0.938 gramme of alkaloid was recovered out of 2.25 grammes injected.

It may be an advantage while referring to the rate of quinine absorption to cite the following experiment.

0.1 gramme of bi-hydrochloride of quinine in 0.25 c.c. saline was injected into the thigh muscles of both hind legs of a guinea-pig; ten minutes later a similar quantity was inoculated into the muscles of the left fore leg, and the animal was killed immediately. The left hind leg was amputated at the hip joint and handed to Captain Ferrey entire. He obtained 0.061 gramme of alkaloid from the tissues which amounts to 0.075 gramme of the bi-hydrochloride. The tissues in the injected areas showed spreading oedema and marked necrosis of muscle. Agglutination of red cells and haemolytic changes had occurred. No tissue reaction was present.

The tissues examined *immediately* after the intra-muscular injection were oedematous and the muscles showed evidence of necrosis.

#### MICROSCOPICAL CHANGES IN THE AFFECTED TISSUES.

*Lesions Nos. 1 and 2.* The tissue changes in the affected muscles which are of eight and seven days standing respectively were similar although quinine alkaloid was injected in the first case and quinine bi-hydrochloride in the second. Microscopically there was typical fibrous myositis with diffuse muscle degeneration. The abundance of nuclei in the muscles among the fragmented fibres was very apparent, and in some instances gave the well-known appearances of pseudo-giant cell formation. Large haemorrhages were observed more especially in relation to strands of necrosis. Mononuclear cells were numerous throughout the new-formed fibrous tissue, and foci of polynuclear phagocytes. The free iron reaction was demonstrated, and spider cells lying in the fibrous tissue were filled with these granules.

*Lesions Nos. 3 and 4* resulted from the injection of quinine bi-hydrochloride on successive days. The fibrous tissue formation was somewhat similar to that which has been referred to in the case of the first two experiments. Large tracts of structureless walls filled with tinged serum were abundant, as also scattered haemorrhages and phagocytosis of red cells by large mononuclear cells. Necrosis of muscle and splitting up of muscle fibres were more evident than in the first two experiments.



*Lesion No. 5.* Extensive necrosis of muscle and "vacuolation" had occurred. Some of the larger blood vessels were thrombosed and complete necrosis of the muscular walls was evident while other vessels showed intense polynuclear inflammation. Strands of polynuclear cells were scattered all through the connective tissue stroma.

*Lesion No. 6.* The most striking feature in this experiment was a zone of necrosed muscle with fibrosis divided from a layer of vacuolated and distorted muscle cells by a broad line of haemorrhage, while acute oedema extended for some distance between the fibres.

*Lesion No. 7.* This lesion showed two distinct pathological changes:

(1) Haemorrhagic area which contained tracts of free blood and congestion of blood vessels together with areas of haemolysed blood in which were present agglutinated red cells. Intense polynuclear inflammation had occurred in the vessel walls and all stages of muscle degeneration.

(2) Necrotic area. The contrast with the vascular area was most marked. Extensive necrosis of muscle with outlying fibres swollen, vacuolated, and distorted and necrosis of the muscular walls of the large blood vessels was found. Throughout the whole tissue there was polynuclear inflammation.

*Lesion No. 8.* This was produced at the end of one hour by one injection of 2.25 grammes of bi-hydrochloride of quinine. Wide tracts of haemorrhage occurred along the muscle bundles with acute oedema spreading in all directions. Muscle fibres were necrosed and in all stages of degeneration. In one portion of the affected muscles wide areas of haemorrhage had occurred with direct destruction of large blood vessels. In this area intense polynuclear inflammation was present while in an adjoining area leucocytic reaction was absent. Rings of polynuclear cells were in evidence in the sheath of the muscle fibres, infiltrating the fibres, and dividing them up into large granular masses. Wide areas of lysed and partly lysed red cells were present. Phagocytosis of red blood corpuscles by large mononuclear cells was very evident, also clumps of agglutinated erythrocytes.

#### FIXATION OF QUININE IN THE TISSUES.

These experiments were undertaken at the suggestion of Captain J. F. Gaskell, R.A.M.C., so as to ascertain whether quinine when injected into the muscles becomes immediately fixed locally, as if so, quinine "absorption" from the tissues would be only an apparent effect.

Experiment 1. Cast Mule. 1.0 gramme of alkaloid quinine was injected as the bi-hydrochloride into the belly of a leg muscle which had been exposed for this purpose. The blood vessels were tied immediately by Captain Moir, A.V.C., and the whole muscle and tendons were removed without delay. The chemical analyses were made by Captain F. S. Hele, M.D., R.A.M.C., and the results were as follows:

Watery extract, 0.217 gramme.

In muscle, 0.595 gramme.

In cloth (used in the experiment), 0·026 gramme.

Total quantity of quinine alkaloid obtained from the muscle, 0·838 gm.

Experiment 2. Similar experiment to No. 1 except that the muscle remained in the body for twenty minutes after the blood vessels were tied by Captain Moir, A.V.C.

Chemical analysis by Captain Hele, R.A.M.C.

Watery extract, 0·307 gramme.

In muscle, 0·460 gramme.

In cloth, 0·029 gramme.

Total quantity of quinine alkaloid extracted from muscle, 0·796 gramme.

The results of these experiments do not suggest that quinine is fixed in the tissues immediately after inoculation to any appreciable extent. Captain Hele considers that the amount "lost" in the above experiments was due to technical difficulties.

#### THE EFFECTS OF INTRA-MUSCULAR INJECTIONS OF QUININE IN ANIMALS RENDERED ANAEMIC.

These experiments were undertaken to ascertain whether intra-muscular injections of quinine produced more severe tissue changes in animals in which a severe haemolytic anaemia had been induced than in control animals. The anaemia and haemoglobinuria were induced by injecting rabbits with the serum of a cat which had been immunised with rabbit's cells for the purpose of these experiments. The immune serum was injected intravenously. Blood counts were made at daily intervals and the weights of the animals were carefully recorded. It was necessary to have records of these data because many patients who receive intra-muscular injections of quinine are anaemic and some are suffering from various grades of haemolytic toxæmia. The most exhaustive experiment will be referred to in detail. Intra-muscular injections of bi-hydrochloride of quinine in saline were given in varying amounts from large to excessive doses with the object of exciting more severe tissue changes in the anaemic animals than in the control. The quinine injections were made previous to and during the period of severe anaemia. There was no leucocytosis. On the contrary a definite reduction in the total white cells occurred, followed by a rise to the total previous to the inoculation as the condition of the blood improved. A full record of the three most important varieties of white cells are given, but the only noteworthy feature is the absolute increase in larger hyaline cells, as met with in malarial fever. The animal showed the effects of each injection, but no more so than normal rabbits which received similar inoculations. When the animal was killed one month from the commencement of the experiment the sites of the quinine inoculations were represented as patches of scar tissue which were most obvious in the case of the excessive dose of 0·6 gramme given thirty days before the death of the animal. The results of the microscopical examination of the scar tissue were similar in



each instance. Fibro-myositis—necrosed muscular tissue—scattered round celled inflammation, well shown as a perivascular effect—and small foci of polynuclear inflammation were the conspicuous features of the various lesions. To quote this experiment is sufficient for the purpose intended, as it is definitely shown that quinine when given by the intra-muscular route in con-

Dates	Weights	Red cells per c.mm.	H.B.	C. I. (Colour- Index)	Leuco- cytes per c.mm.	Differential count of leucocytes. 1st column = %, 2nd column = no. per c.mm.										Doses of quinine bi-hydrochloride in saline. Total bulk in- jected on each occasion = 1 c.c.
						Nucleated red cells, No. % of leucocytes	Finely granular oxypill	1	2	1	2	1	2	Large lymphocytes	1	
29/5/18	1510 grms.	6,000,000	100 %	0.8	7500	0	21.9	1642	76.0	5700	0	0	0	0	0	0
30/5/18	1490 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
31/5/18	1490 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/6/18	1510 "	6,000,000	100 %	0.8	6400	0	32.6	2086	66.0	4224	0.6	38	0.6	0.45	0.25	gramme
2/6/18	1515 "	—	88 %	—	—	0	13.0	—	85.3	—	1.0	—	—	—	—	—
3/6/18	1445 "	4,300,000	50 %	0.5	7200	6.0	20.0	1440	79.3	5709	0	0	0	0	0	0
4/6/18	1400 "	1,500,000	32 %	1.0	5000	7.0	27.0	1350	72.3	3615	0.6	30	0.09	0.45	0.09	0.09
5/6/18	1400 "	1,500,000	32 %	1.0	4500	2.0	34.0	1530	65.3	2938	0	0	0	0	0	0
6/6/18	1390 "	2,000,000	36 %	0.9	5200	1.0	55.0	2860	44.6	2319	0	0	0	0.045	0.15	0.15
7/6/18	1300 "	2,056,250	40 %	1.0	5400	4.0	34.3	1812	56.6	3056	4.3	232	0	0	0.15	0.15
8/6/18	1330 "	2,170,000	35 %	0.8	5000	16.5	60.0	3000	40.0	2000	0	0	0	0	0.15	0.15
9/6/18	1335 "	3,280,000	65 %	1.0	4900	10.0	30.0	1470	69.0	3381	1.0	49	0.45	0.15	0.15	0.15
10/6/18	1365 "	3,290,000	65 %	0.9	6800	2.5	32.0	2176	67.5	4590	0.5	34	0.45	0.15	0.15	0.15
11/6/18	1320 "	4,200,000	66 %	0.7	7260	2.0	42.0	3024	55.0	3960	2.0	144	0.45	0.15	0.15	0.15
12/6/18	1350 "	4,290,000	65 %	0.7	7280	2.0	29.0	1988	66.0	4752	5.0	360	0.45	0.15	0.15	0.15
30/6/18	1610 "	4,496,875	82 %	0.9	7220	0	31.0	1532	58.0	4176	9.0	648	0.45	0.15	0.15	0.15

centrated and massive doses excites no greater reaction in the tissue of animals rendered anaemic than in the case of the normal, while no general effect occurred even when the inoculations were made on successive days. Other experiments completed on these lines led to similar conclusions. One rabbit,

in which a severe experimental anaemia had been induced, received two massive intra-muscular doses of quinine during the period of haemoglobinuria, but the effects were similar to those obtained in the control animals, while a diminution in the total leucocytes was recorded.

#### HUMAN MUSCLE.

Muscle tissue from the region of a previous quinine inoculation has been examined from several cases in this Command. The interval between the inoculation and the examination varied from so short a period as one hour up to three months. It has been suggested that the effects of quinine on the tissues is not capable of demonstration until twenty-four hours from the time of the inoculation. This view is erroneous on experimental evidence while it is equally fallacious in the case of a human subject. A man, comatose from malarial fever, was admitted to hospital, an intra-muscular injection of twenty grains of bi-hydrochloride of quinine was given, but the patient died one hour later. At the autopsy a large area of black green necrosis about four by four inches surrounded by gelatinous oedema was discovered at the seat of inoculation. All cases which I have had the opportunity to examine have received concentrated quinine. Experimentally, quinine injections can be given and the autopsy performed immediately, but the neurotic action is quite obvious. No detailed description of the microscopical changes will be given in this section except such as refer to points of special importance. Certain cases of special interest will be briefly referred to so as to illustrate the most essential features as regards quinine inoculation. Twelve grains of bi-hydrochloride of quinine were injected into the right buttock forty-eight hours before death. At the autopsy a large area of complete necrosis of muscle was observed together with a wide tract of haemorrhage due, as was proved on microscopical examination, to complete destruction of the wall of a large artery. The entire necrosed muscular tissue together with that tissue in immediate contact was examined chemically, except for a small portion reserved for microscopy, but no quinine was detected. Two intra-muscular injections of fifteen and twenty grains respectively had been made at an interval of twenty-four hours and death occurred about twenty hours later. The resulting lesion was similar in each case—large area of necrosis, a band of haemorrhage and congestion, with a wide tract of gelatinous oedema.

In the case of a Greek labourer who had received an intra-muscular injection of fifteen grains of bi-hydrochloride of quinine twenty-two hours before death, only 0·02 gramme of the alkaloid was obtained from a large area of necrosis at the seat of the inoculation. The absorption here was rapid in spite of the fact that the patient was dying from fulminating pneumococcal septicaemia.

Certain cases of malarial fever with malarial parasites present in the circulating blood were complicated with blackwater fever, but the effects from intra-muscular injections of bi-hydrochloride of quinine were similar to those



observed in the uncomplicated cases, except that the haemorrhagic zone appeared darker in colour, almost black in some instances (Pl. III, Fig. 6).

One of the most important cases which illustrate the action of quinine occurred in the case of a man who died from blackwater fever and malaria. Four days before death he received an intra-muscular injection of twenty grains of bi-hydrochloride of quinine. The inoculation was made into the same buttock which had been injected three months previously with a similar dose for malarial fever. This man had complained of aching and cramping pains in the buttock at the site of the inoculation, especially if he sat for any length of time on a hard seat or had prolonged exercise. At the autopsy intense necrosis had occurred at the site of the recent injection which merged into the old lesion, which showed dense fibrous tissue at the centre of the focus, and a definite broad band of fibrous myositis at the periphery. Acute polynuclear inflammation from the recent injection had extended to this layer of fibrous myositis. The pain referred to could be explained by the patch of dense fibrous tissue in the centre of an important muscle surrounded by a zone of fibrous myositis, but further, owing to a fibro-neuritis which existed in this area. Some of the nerves of the muscle were surrounded by dense fibrous tissue, and similar foreign tissue had replaced many of the nerve fibres. Similar examples were met with in cases of much shorter duration, and also experimentally, a fibro-myositis, and fibro-neuritis.

A patient was admitted to hospital with malignant malaria. He received an intra-muscular injection of 1.33 grammes of bi-hydrochloride of quinine but died two hours later. The whole of the necrotic tissue, which was three inches square, surrounded by oedema, was removed for chemical investigation except for a small portion reserved for microscopy. There was extensive necrosis of muscle, necrosis of the walls of blood vessels, marked evidence of haemolysis, but no acute inflammation was present.

Although the patient was inoculated when *in extremis*, only 0.344 gramme of alkaloidal quinine was extracted from the tissues.

The fact that necrosis of the tissues always accompanies the intra-muscular or subcutaneous injections of quinine is not realised sufficiently by Medical Officers, even those who have employed these methods on a large scale. No better illustration of the correctness of this statement can be furnished, than by quoting the following instance. Owing to certain bad results which had occurred from intra-muscular injections of quinine, an Army order was issued to the effect that all Divisional Officers must report in detail to the D.M.S. any ill effects subsequent to intra-muscular injections of quinine. One Divisional Officer after fifteen months' experience of this method of treatment of malaria furnished a report to the D.M.S. to the effect that a man had died from malaria and at the post-mortem examination a wide area of necrosis of muscle was found at the seat of injection. He concluded his evidence with the statement that all Medical Officers in his unit had been warned of this unfortunate incident and that every effort would be made to prevent a

recurrence of this disaster! I have discussed the question of quinine necrosis with innumerable Medical Officers who have had wide experience of intra-muscular injections of quinine and it is by no means uncommon to learn from them that they were unaware that such effects occurred in the tissues apart from negligence. It is this lack of knowledge of the methods of quinine administration which serves to explain the cause of many of the disasters which have occurred. It is, therefore, necessary to emphasize that quinine injections should not be given in the vicinity of large nerve trunks, or main arteries, that the injections should not be repeated in the same area of muscular tissue and that this method of quinine administration should only be employed when circumstances demand it<sup>1</sup>.

The chief complications of intra-muscular injections of quinine in the human subject during 1916-17 and '18 of which I have records were as follows: (1) Tetanus, (2) Gangrene, (3) Abscess formation, (4) Pyaemia, (5) Nerve Palsies, (6) Haemorrhage from large Arteries, (7) Sciatica, (8) Chronic muscular pain, (9) Pain and deficient movements in affected muscles, (10) Thrombosis in varicose veins.

#### THE RESULTS OF THE ESTIMATION OF QUININE ALKALOID IN HUMAN MUSCLE SUBSEQUENT TO QUININE INJECTIONS.

No. 1, Broncho-pneumonia. Intra-muscular injections of 21 grains of bi-hydrochloride of quinine into left buttock. Two days later 26 grains were injected on the opposite side. Death on the following day. Amount of quinine alkaloid recovered from first injection, 0.227 gramme, and from the second injection, 0.345 gramme.

No. 2, Malignant malaria. Intra-muscular injection of 15 grains of bi-hydrochloride of quinine. Death four days later. Amount of quinine alkaloid recovered, 0.002 gramme. This patient had received ten intra-muscular injections in fourteen days amounting to 115 grains, and six intra-venous injections which totalled 55 grains.

No. 3, Malignant malaria. Intra-muscular injection of 10 grains of bi-hydrochloride of quinine. Death three days later. Amount of quinine alkaloid recovered from muscle, 0.0115 gramme.

No. 4, Lobar pneumonia. Intra-muscular injection of 15 grains of bi-hydrochloride of quinine. Death 24 hours later. Amount of quinine alkaloid recovered from muscle, 0.048 gramme.

No. 5, Malignant malaria. Intra-muscular injection of 18 grains of sulphate of quinine. Ten days later large quantity of pus evacuated. From 50 c.c. of the pus, 0.0012 gramme of quinine alkaloid was obtained.

No. 6, Malignant malaria. Intra-muscular injection of 10 grains of bi-hydrochloride of quinine. Death 26 hours later. Amount of quinine alkaloid recovered from muscle, 0.041 gramme.

<sup>1</sup> The oral method of administration of quinine is greatly neglected by some medical officers who are placed in charge of cases of malarial fever.



No. 7, Malignant malaria. Intra-muscular injection of 20 grains of bi-hydrochloride of quinine. Death 2 hours later. Amount of quinine alkaloid recovered from muscle, 0.344 gramme.

No. 8, Lobar pneumonia, Pneumococcal meningitis. Intra-muscular injection of 15 grains of bi-hydrochloride of quinine. Death 20 hours later. Amount of quinine alkaloid recovered from muscles, 0.0215 gramme.

In every instance the quinine was injected into the muscles in concentrated solution—the total quantity of fluid injected did not amount to more than a few c.c. The results show, however, that the absorption was rapid, as only traces of quinine were recovered from the entire lesions, apart from a small focus which was reserved for microscopy, and from the surrounding muscular tissue. In No. 7, 20 grains of the bi-hydrochloride of quinine were injected into the muscles, and the patient died two hours later, but only 0.3 gramme was recovered. In each instance the area of necrosis was considerable, and the tissues showed very marked changes on microscopical examination.

#### AN EXAMINATION OF MUSCLE TISSUE NECROSED FROM QUININE INJECTIONS FOR THE PRESENCE OF MALARIAL PARASITES.

Certain specimens of muscular tissue which had been examined for the presence of malarial parasites subsequent to the injections of quinine showed most unexpected results. The blood cells in the necrotic vessels were completely haemolysed, yet malarial parasites were present in some instances in relatively large numbers and the bodies of the parasites were well stained. The parasites would be diminished in numbers as compared with the control muscle from another area of the body or from some visceral lesions, but perfectly staining parasites were found lying in a necrosed vessel in which there were no normal red cells. It is strong evidence that destruction of red cells and tissues generally is much more readily excited by quinine solutions than malarial parasites.

One case will be referred to in detail to emphasize this fact:

##### *Malignant Malaria.*

Blood film showed a very heavy infection of red cells with the ring form of parasites. Sporulating parasites were also numerous. Time 10.30 a.m.

20 grains of bi-hydrochloride of quinine were injected intra-muscularly at 11 a.m. and 20 grains were injected intra-venously. Death at 3.45 p.m. on same day.

Wide areas of necrosis of muscle at the seat of the quinine injection existed. There was great destruction of the walls of blood vessels and the blood cells were completely lysed, while the muscle tissue itself showed considerable necrosis. Large dot parasites with well-stained bodies and abundance of pigment were relatively numerous lying among the red cell *débris*. Parasites

however were far more numerous in the internal organs and in other muscular areas.

#### INTRA-MUSCULAR INJECTION INTO FROGS.

Several frogs received intra-muscular injections of quinine bi-hydrochloride in saline in suitable doses for body weight, and were killed three hours later. The muscles at the sites of the quinine injections were opaque. There was marked oedema, complete necrosis of muscle fibres, other fibres vacuolated or represented as granular masses and haemolysis of red cells in the affected areas.

Four frogs with an average weight of 60 grammes were injected (I.M.) with quinine and the resulting lesions were as follows:

1. Frog injected with 0.0004 gramme of quinine bi-hydrochloride (0.1 c.c.) and killed 22 hours later. Muscles opaque and showed extensive necrosis.

2. Frog injected with 0.0004 gramme of alkaloid in 0.02 c.c. of ether and killed 22 hours later. Extensive muscular necrosis. Slight inflammatory reaction.

3. Quinine injection as in case of frog 1. Animal killed 3 days later. Extensive muscular necrosis—abundant haemorrhages—and widespread inflammation were very evident.

4. Frog injected with 0.0004 gramme of the alkaloid suspended in saline and killed 3 days later. Results similar to Experiment 3.

These results were similar to those obtained in the case of the warm-blooded animals.

#### CONCLUSIONS.

(1) Concentrated preparations of quinine produce more intense necrosis than dilute, but dilute preparations such as are of practical utility excite oedema and necrosis at the seat of inoculation. The difference between these two methods of quinine inoculation is not of sufficient value to justify active opposition to the method commonly employed.

Inoculation of quinine in solutions so dilute as to avoid oedema and tissue necrosis is not of practical utility in the human subject.

(2) A concentrated solution of quinine is absorbed rapidly from the tissues as shown by chemical analysis even in patients who are *in extremis*. It is not apparently stored as such in liver, kidneys, or heart muscle.

(3) It is essential to realise that tissue necrosis—spreading oedema and local blood destruction—are produced by the solvents employed for quinine administration and the effects are only slightly inferior to those excited by quinine salts and the alkaloid.

(4) No advantage was obtained by the addition of olive oil or fat or by injecting the alkaloid dissolved in alcohol, or ether, whether in concentrated or in a dilute solution.

(5) Tissue necrosis occurs immediately and persists for a considerable period. In some instances the fibro-myositis which results is associated with



a fibro-neuritis which causes various symptoms definitely related to the pathological processes.

(6) Necrosis of blood vessels in the area of inoculation is a common result. This leads to small haemorrhages into the tissues, and has caused severe haemorrhages in the human subject, and experimentally, from rupture of a large vessel. The destruction of the vessel wall is associated with an accompanying thrombosis.

(7) An extensive necrosis produced by an intra-muscular injection of quinine, in the neighbourhood of an important nerve trunk; may result in nerve palsy. Experimentally, complete degeneration of the great sciatic and other nerves has been produced apart from any direct injury to the nerve at the time of the inoculation. In the human subject this disastrous result may be due to spreading oedema and extensive tissue necrosis.

(8) Experimentally, no leucocytosis has ever occurred from quinine injections; on the other hand a leucopenia may develop while an increase of large hyaline cells has been recorded on several occasions.

(9) No essential differences in the degree of tissue necrosis from intra-muscular injections of quinine in malarial fever or malarial fever associated with blackwater fever were observed.

(10) Repeated intra-muscular injections of quinine should not be given into the same area of muscle, or tissue directly adjacent, as otherwise permanent injury of muscle<sup>1</sup> or nerves may occur.

#### ACKNOWLEDGMENTS.

To Captain C. E. C. Ferrey, O.B.E., R.A.M.C. (T.F.), Analytical Chemist to the Central Laboratory of the B.S.F., I am greatly indebted for the whole of the chemical analysis of the tissues for the determination of the presence and amount of quinine. This investigation, which has been a laborious process, is of the utmost value in association with the histological findings.

To Captain Moir, A.V.C., Bacteriologist to the A.V.C., B.S.F., I am indebted for considerable help in the investigation of the cast mules and horses which were inoculated with quinine.

Captain A. Wilkin, R.A.M.C., rendered me valuable assistance during the process of the work.

The tissue from human sources has been procured for me by various bacteriologists in the Command, to whom my thanks are due.

To Corporal P. Panichelli, M.S.M., R.A.M.C. (T.F.), I am indebted for the illustrations which serve to explain what the text is incapable of defining.

<sup>1</sup> The gluteal regions, obtained from a man who had daily intra-muscular injections of quinine, nine in all, were shown at the British Medical Association meeting in London, 1919. As a result of the injections wide tracts of muscle were necrosed and only fragments of healthy tissue remained.

## DESCRIPTION OF PLATE III.

- Fig. 1. *Quinine rabbit*, 53-(4). Section of muscle from a rabbit which had received an intra-muscular injection of 0.04 gramme of alkaloid quinine in 1 c.c. of alcohol four days before death.
- Fig. 2. Agglutination of red blood corpuscles in a large vessel in the tissue of a horse which had been injected with 1.1 gramme of bi-hydrochloride of quinine 30 hours previously.
- Fig. 3. Complete necrosis of large artery as a result of an intra-muscular injection of quinine alkaloid in brandy.
- Fig. 4. Section of great sciatic nerve in a rabbit which had received an intra-muscular injection of 1 grain of quinine alkaloid in alcohol 11 days previously.
- Fig. 5. Muscle from a mule which had received 2.25 grammes of bi-hydrochloride of quinine in 5 c.c. of saline five days before death. Natural size.
- A. Oedema, necrosis, patches of haemorrhage.
  - B. Haemorrhagic line showing intense inflammation.
  - C. Oedematous muscle.
- Fig. 6. Portion of human muscle from a case of Blackwater Fever and Malaria. Patient had received an intra-muscular injection of 20 grains of bi-hydrochloride of quinine 48 hours before death. Natural size.





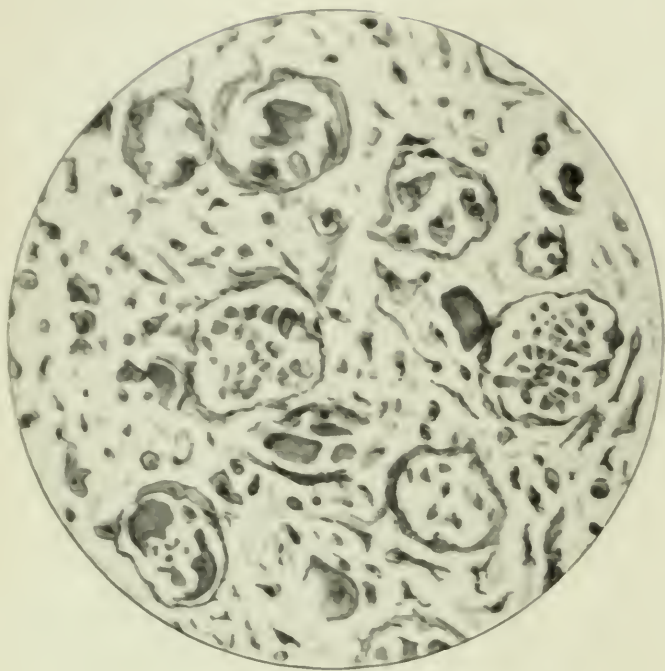


Fig. 1

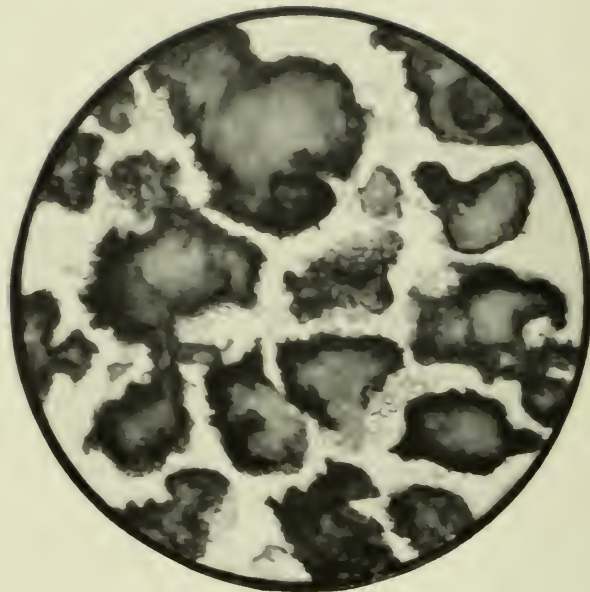


Fig. 2

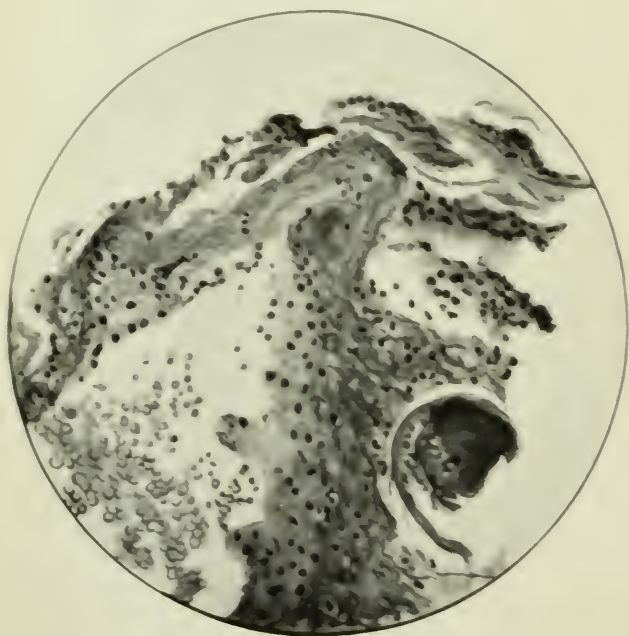


Fig. 3

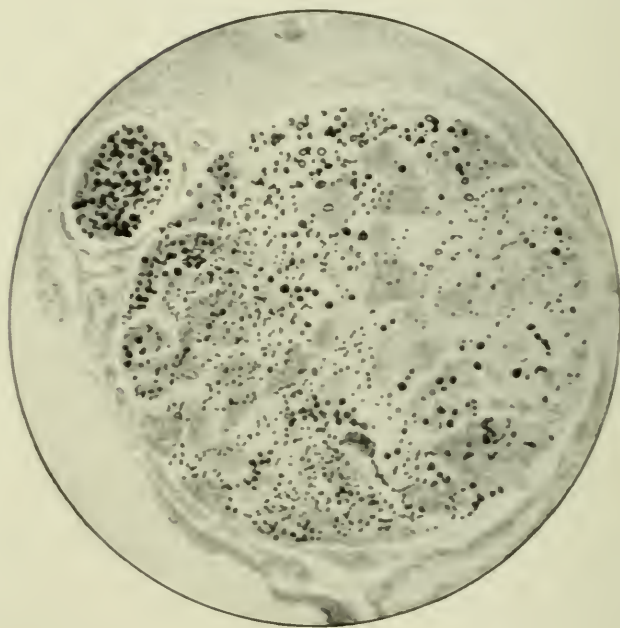


Fig. 4





Fig. 5



Fig. 6





STUDIES ON THE ACTION OF ELECTROLYTES  
ON BACTERIA.

PART I.

THE ACTION OF MONOVALENT AND DIVALENT SALTS ON THE  
CONDUCTIVITY OF BACTERIAL EMULSIONS.

By C. SHEARER, F.R.S.

(From the Pathological Laboratory, University of Cambridge.)

(With 8 Charts.)

CONTENTS.

	PAGE
I. Introduction . . . . .	337
II. Description of Experiments . . . . .	341
III. Discussion of the bearing of some of the results of the foregoing experiments on the rôle of certain salts in the question of wound infection . . . . .	352
IV. Summary . . . . .	359

I. INTRODUCTION.

To the bacteriologist the problem of how chemical substances enter and leave the cell presents many features of special interest. It is well known that singularly few bacteria produce true exotoxins, the majority forming endo-  
toxins, but we possess little information as to the conditions under which these pass the cell-wall. In some instances it would seem that some form of autolysis enters in the matter to a considerable extent<sup>1</sup>.

In the case of the meningococcus, Gordon (1918) has pointed out that different strains of this germ possess widely different toxic powers for mice, but that they all have the same minimal lethal dose for these animals when ground up and extracted with distilled water. Thus in this case it would look as if toxicity depended on the ease with which the endotoxin diffuses out of the cell; while some races of the germ allow it to escape freely, others only permit it to pass in small quantities, but that all strains possess the same amount. Similar conditions probably hold for many other pathogenic bacteria.

The striking part played by capsule formation in the acquisition of viru-  
lence by many races of bacteria clearly points to the importance of this struc-

<sup>1</sup> These studies had their origin in an attempt to determine some of the conditions of autolysis in the meningococcus.

ture in preventing the penetration of many substances into the bacterial cell. Danysz (1900) has drawn attention to the fact that anthrax bacilli frequently possess arsenic and serum resistant qualities which are always coupled with their ability to form capsules. It has been shown that organisms like the pneumococcus and some streptococci, which sometimes acquire this power, are invariably most virulent in the capsulated condition. The capsule would seem to play a rôle similar to the action of one colloid on another, in preventing aggregation and flocculation, and the notorious difficulty always experienced in agglutinating these forms amply confirms this contention. This is supported by Porges's (1905) demonstration that they are easily agglutinable, if the capsules are first removed by heating a short time in weak acid.

It is clear that the question as to how endosmosis and exosmosis takes place in living bacteria is one of fundamental importance, and its solution is bound up with many questions of infection, virulence and immunity. It is remarkable that its investigation so far has attracted little attention from bacteriologists. If we turn to the domain of general physiology, we find a large and ever-growing literature dealing with the subject. It has been attacked from many sides, and the application to its study of some of the recent discoveries of colloid and physical chemistry have added very much to our knowledge, and our methods of dealing with the problem have been greatly extended and improved.

The question is necessarily a complex one, in which a large number of related and interdependent actions are simultaneously taking place. It involves the passage of substances from the external medium, through the cell-wall to the cytoplasm, across the intermediary boundary phases. In a balanced solution, that is, one that contains two or more salts in definite proportions, so that their specific individual action does not come into play, but is antagonised, living cells can remain for relatively long periods without suffering any injury. If the salts are sufficiently numerous, such solutions can even act as nutrient fluids, in which many plants and bacteria can actively grow and reproduce. It has been pointed out that under these conditions the cell-wall must be semi-permeable, that the passage through it of salts must be conditioned to a large extent by the chemical changes going on within the cell, and that the physical properties of the cell-wall play a minor part in the matter. Regarded in this light the problem is a dynamical one, in which equilibrium is constantly being adjusted between the external and the internal conditions.

If we place living cells, on the other hand, in weak but pure solutions of these salts we find we are dealing with a different problem. The fundamental conditions are now altered and the normal stability of the cell-wall has been destroyed. In such a solution we are studying the unantagonised action of a salt on the cytoplasm.

The results obtained from the investigation of this side of the question are of great experimental interest. It is particularly interesting to apply



experiments of this kind to bacteria, as there is no other class of organisms in which surface conditions and the properties of the cell-wall play a greater part, as they present relatively such a great extent of surface for a given mass, on account of their small size, surface conditions on all occasions determine their behaviour. The following paper for these reasons is mainly devoted to an investigation of the action of various salts in pure solutions in altering the cell-wall and surface conditions of bacteria.

It has been shown by Oker-Blom (1900), that if we estimate the relative conductivity of various mixtures of sand and a solution of NaCl, we find this is proportional to the quantity of sand in the solution. As an electric current is carried through a solution by its ions, the rate at which these will conduct the current is dependent on two factors, first the potential gradient, and secondly the friction or resistance offered by the solution to the passage of the ions. If the potential gradient is kept fixed, then any additional obstruction to the passage of the ions will necessarily increase the resistance and lower the conductivity. In a mixture of 61 parts quartz sand and 39 parts NaCl solution, he found the conductivity was 24·5 per cent. that of the NaCl solution without sand. The ions of such a mixture were forced by the grains of sand to take a zigzag course in passing from one electrode to another, and the resistance is proportionally increased.

In a similar manner the conductivity method has been used by Róth (1897), Bugarsky and Tangl (1897); Oker-Blom (1900), Stewart (1899), Woelfel (1908) to determine the proportion of blood corpuscles to plasma. If we measure the comparative conductivity of blood serum, whole blood, and corpuscles alone, we find the plasma has the highest figure, the corpuscles the lowest, and the whole blood an intermediate position. Thus these workers conclude, that the cellular elements of the blood, like the sand grains in the previous instance, offer considerable resistance to the passage of the ions of the plasma.

Stewart (1910), as the result of extensive studies on the conductivity of normal as compared with laked blood, comes to the conclusion that the cell "envelopes" of the corpuscles are relatively impermeable to the ions with which they are normally in contact. He found, moreover, that any strong cytolytic agent, such as saponin, which rapidly destroys the enveloping membrane, at once increases the conductivity of the corpuscles.

This conclusion would seem to be supported by Höber's (1913) experiments, where he has shown that if a conducting body is placed in the axis of a coil of wire, through which a rapidly alternating current is being passed, it will dampen or diminish this current in proportion to its power of conductance. The internal conductivity of blood cells is therefore greater than their conductivity as determined in the ordinary way. This indicates that the cell-wall or plasma membrane of the corpuscles offers considerable resistance to the passage of ions. He also found that saponin, which undergoes no dissociation, has little effect on the internal conductivity, while its cytolytic

action on the cell-wall greatly increases conductivity as determined by the Kohlrausch method; thus confirming Stewart's previous discovery.

If instead of sand grains or blood cells we add living bacteria to a clear solution of Ringer's fluid, we find in a similar way, that the resistance will increase and the conductivity decrease in proportion to the number of bacteria added. If sufficient bacteria are added to turn the fluid a white milky colour, the resistance is usually double that of the clear solution; if we add enough bacteria to turn the mixture into a thick paste the resistance may be trebled, while if we centrifuge the germs down in a solid mass, the resistance of the bacterial deposit will now be five or six times that of the original plain fluid. By placing bacteria in a similar manner in various salt solutions, if we take the precaution to make these solutions of the same conductivity as the Ringer's solution, we can determine the specific action of these solutions in altering the normal conductivity of the bacterial cell as originally determined in the Ringer's solution.

The work of Osterhout (1913), McClendon (1910), and Gray (1916) has shown that the Kohlrausch conductivity method is readily applicable to the study of the action of salts, in this manner, on living plant tissues and the animal egg-cell. Moreover the work of Perrin (1904), Girard (1910), (1919 a), (1919 b), Mines (1911) and Brooks (1917), on the passage of electrolytes through artificial and natural membranes, form a series of researches of remarkable interest, when compared with the results obtained by the conductivity method.

The conductivity method seems to have been first employed in the investigation of living cells by a number of independent workers about the same time. Among these are Róth (1897), Bugarsky and Tangl (1897), and Stewart (1910). Stewart found as already mentioned that the conductivity of blood plasma was greater than that of the whole blood, and that the resistance rose rapidly with an increase in the number of corpuscles. The action of saponin, in lowering the resistance of the corpuscles, was noted and he drew attention to the fact that it produced this effect as well on dead corpuscles. McClendon (1910) was the first to apply the method, using special electrodes, to the estimation of the changes taking place in the conductivity of the Echinoderm egg on fertilisation. This work was elaborated still further by Gray (1916), who demonstrated the marked action of the trivalent salts in altering the conductivity of these eggs in sea-water. The method was applied about the same time independently by Osterhout (1915), to determine the conductivity of the tissues of the marine alga *Laminaria* to salts in pure and balanced solutions.

It was found difficult working with bacteria to obtain resistances as high, even with the thickest emulsions or solid masses of bacteria, as those obtained by these workers. Osterhout (1918) using a special apparatus, and placing a large number of discs of *Laminaria* tissue one against another, like a roll of coins, was able to obtain resistances well over a thousand ohms; while Gray, using eggs that had been specially washed to remove the jelly-like



outer membrane, obtained resistances of 250–350 ohms, while the resistance of the same quantity of sea-water under the same conditions was only 16 ohms.

## II. DESCRIPTION OF EXPERIMENTS.

It was found possible, using bacteria made up into thick emulsions, to obtain very similar consecutive readings of their resistances, if after three or four preliminary washings in Ringer's fluid they were centrifuged down into a solid mass, and then made up in a thick paste with the same quantity of fresh fluid each time. In the following experiment (Table I) is given a

Table I.

Experiment giving a series of consecutive readings of resistances made on the same meningococcus emulsion, in Ringer's solution. The bacteria were centrifuged down into a solid mass each time and then made into a thick paste with the same quantity of Ringer's sol. Temp. 25° C. Cell constant =  $4.22 \times 10^{-1}$ .

1st time	104 ohms resistance	6th time	108 ohms resistance
2nd	106	7th	108
3rd	105	8th	107
4th	106	9th	107
5th	108	10th	108

series of ten consecutive readings taken in this way, on the same emulsion of the meningococcus. It will be seen that they agree with one another very well, and only differ within a margin of a few ohms. There is a slight tendency for the resistance to rise slightly towards the end of the experiment. This is doubtless due to the washing away of salts brought over with the germs, despite the preliminary washings from the culture medium. It can be neglected as it is always slight, and the resistances for the most part in the following experiments have to deal with a fall not a rise. Only fresh 24 hrs. cultures were used, 24–30 plates of tryptagar being sufficient to furnish enough material for one experiment. The bacteria were washed off the plates in a large quantity of neutral Ringer's solution<sup>1</sup> and centrifuged down and rewashed three times in succession in considerable quantities of fresh fluid each time, before being used for any of the experiments as already mentioned.

All measurements were made in a thermostat tank, at a fixed temperature of 25° C., which did not vary more than a twentieth of a degree. A direct reading Kohlrausch bridge was employed. In the earlier experiments the resistances were determined with a large Hamburger cell. This was made to fit into the tubes of the centrifuge direct and the emulsions were centrifuged down in the conductivity cell itself. In this way it was possible to get particularly thick emulsions having about 110 ohms resistance, while the same quantity of Ringer's solution had only 26 ohms resistance. In the later experiments a much smaller cell was employed and the emulsions were made

<sup>1</sup> M/8 KCl	...	25 c.c.	0.0031 M.	0.024 % KCl.
CaCl <sub>2</sub>	...	15	0.00187 M.	0.0208 % CaCl <sub>2</sub> .
NaCl to	...	1000	0.12 M.	0.7 % NaCl.

considerably thinner, which gave slightly more uniform readings, and also saved time in the preparation of the material itself. In this case resistances of 150-200 ohms could be obtained, while the same quantity of Ringer's solution had about 85 ohms resistance. The cell constants for these cells are given in the tables giving the data of the various experiments<sup>1</sup>.

If sufficient care was taken to get the original emulsion fairly thick, resistances of 110 ohms could be pretty constantly obtained with the meningococcus and slightly higher resistances with *B. coli*. The same quantity of Ringer's solution in the same cell under the same conditions of temperature having about 26.7-37 ohms resistance. Thus about three-quarters (or a little less) of the resistance of the above solution is due to the presence of the living bacteria. It was found that dead bacteria offer no resistance to the passage of the ions of a solution. Emulsions of dead bacteria have almost the same resistance as that of the fluids in which they are suspended. If the above emulsion of bacteria in Ringer's solution were killed by adding a drop of formalin or warming the emulsion to 55° C. for a few minutes, then on washing the emulsion several times in fresh changes of Ringer's solution to get rid of the formalin or of any salts derived from the dead bacteria, it will be found that the resistance has fallen from 104 or 108 ohms to 26.7 or 30 ohms, the resistance of the Ringer's solution alone. Thus there would seem to be something about the living condition which produces the resistance, and that dead cells offer no more than would so much agar or gelatine.

Living bacterial emulsions undergo little change in conductivity on standing for several days in Ringer's solution, showing a slight tendency to fall on account of adsorption of a little of the salts by the bacteria; this fall is always slight.

If, however, we make up the bacterial emulsion in pure NaCl\* instead of Ringer's solution, the NaCl employed having the same conductivity as that of the Ringer's solution, *i.e.* one in which the resistance is 26.7 ohms (which is not very far removed in strength from a 0.85 per cent. NaCl solution or 0.124 M.), we obtain as in the case of the same emulsion in Ringer's solution an initial resistance of 110 ohms. This gradually drops within a short time and at the end of 30 or 40 minutes becomes the same as that of the NaCl solution without bacteria, *i.e.* 26.7 ohms. (See Table II A and Curve I.)

Thus pure NaCl of about the same concentration as that present in the blood gradually destroys the resistance offered by the bacterial cell. If the germs are allowed to lie in the NaCl for several hours, it will be found that at the end of this time they are dead. If they only remain in the NaCl a short

<sup>1</sup> Unfortunately in the case of the large cell this was not determined until most of the experiments had been made and the electrodes had been damaged by a slight accident. The cell constant was determined after the cell had been repaired and only applies approximately to this cell.

\* All solutions used in the following experiments were made from water that had been distilled three times from glass, and Kahlbaum salts were employed.



time, and are then transferred to Ringer's solution again, they immediately regain their normal resistance and suffer no injury.

In the case of *B. coli* even at the end of one hour in NaCl they are seen to be actively motile, and death only takes place slowly after some time, as the probable result of the gradual diffusion out of the cell of some of the essential chemical substances necessary to the living state.

Table II A.

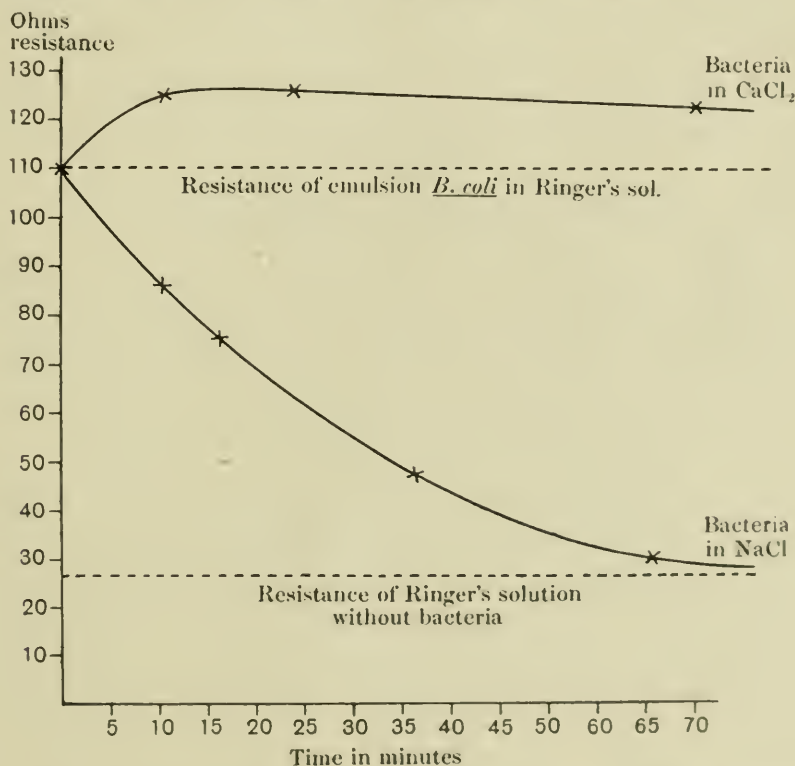
Sodium chloride Experiment.

Temp. 25° C. Resistance constant of cell =  $4.22 \times 10^{-1}$ .

1.	Resistance of Ringer's and NaCl (0.124 M.) each	...	26.7 ohms.
2.	Resistance of Type III Meningococcus emulsion in Ringer	110	ohms.
3.	Resistance due to bacteria, $110 - 26.7$	...	83.3 ohms.
4.	Resistance of same emulsion in NaCl of the same conductivity as Ringer's sol. after		
	10 min.	...	90 ohms.
	15	...	80
	35	...	49
	65	...	28

At the end of the experiment the emulsion was subcultured, at the end of 48 hrs. little growth had taken place, showing the emulsion at the time of subculture was practically dead.

Curve I, showing the resistance of emulsion of *B. coli* in pure  $\text{CaCl}_2$  and NaCl solution of the same conductivity as neutral Ringer's solution. Temp. 25° C. Resistance of all solutions 26.7 ohms. Cell constant =  $4.22 \times 10^{-1}$ .  $\text{CaCl}_2 = 0.1$  M.,  $\text{NaCl} = 0.124$  M.



If, when the resistance of the bacterial emulsion has fallen in the NaCl solution, a little trace of  $\text{CaCl}_2$  is added, it again returns to its normal conductivity, and suffers no injury. Thus the  $\text{CaCl}_2$  antagonises the action of the NaCl.

*Action of Electrolytes on Bacteria*

It was found that a small trace of  $\text{SrCl}_2$ ,  $\text{BaCl}_2$ ,  $\text{CdCl}_2$  could also antagonise in a similar manner the action of  $\text{NaCl}$ . (See Tables and Curves of these experiments.)

Table II B.

Calcium chloride Experiment I. Temp.  $25^\circ \text{C}$ .Cell constant  $= 4.22 \times 10^{-1}$ .

1. Resistance of Ringer's and $\text{CaCl}_2$ (0.1 M.) <sup>1</sup> each ...	...	26.7 ohms.
2. Resistance of emulsion of <i>B. coli</i> in Ringer's sol. ...	...	120.0
3. Resistance due to bacteria, 120 - 26.7 ...	...	93.3
4. Resistance of same emulsion washed in 3 changes of $\text{CaCl}_2$ of the same conductivity as Ringer's sol. after		
10 min. ...	124 ohms.	
25 ...	124	
2 hrs. ...	120	

Emulsion grew well on subculture at end of experiment.

Table III.

Calcium chloride Experiment II. Temp.  $25^\circ \text{C}$ .Cell constant  $= 9.5 \times 10^{-1}$ .

1. Resistance of Ringer and $\text{CaCl}_2$ solutions each ...	...	85.0 ohms.
2. Resistance of emulsion of <i>B. coli</i> in Ringer ...	...	135.0
3. Resistance due to bacteria, 135 - 85 ...	...	50.0
4. Resistance of same emulsion in $\text{CaCl}_2$ sol. (0.1 M.) after		
25 min. ...	167 ohms.	
30 ...	161	
35 ...	161	
2 hrs. ...	161	

Emulsion grew well at end of experiment on subculture.

Table IV.

Calcium nitrate Experiment. Temp.  $25^\circ \text{C}$ .Cell constant  $= 9.5 \times 10^{-1}$ .

1. Resistance of Ringer and calcium nitrate sols. each ...	...	85.0 ohms.
2. Resistance of emulsion of <i>B. coli</i> in Ringer ...	...	120.0
3. Resistance due to bacteria, 120 - 85 ...	...	35.0
4. Resistance of same emulsion in $\text{CaNO}_3$ isotonic with Ringer	124.0	
5. Resistance of same emulsion in $\text{CaNO}_3$ after 1 hour ...	123.0	

Emulsion grew well at end of experiment on subculture.

Further experiments with other salts of the monovalent group, such as  $\text{KCl}$ ,  $\text{LiCl}$ ,  $\text{RbCl}$ ,  $\text{CsCl}$ , showed that each of these in turn possessed in a varying degree the power of reducing the resistance offered to the passage of the ions of a solution, in a manner similar to  $\text{NaCl}$ . In all instances this increase of conductivity was reversible, the bacteria returning to their normal condition on being transferred to any balanced solution, such as Ringer's, sea-water, or van't Hoff's solution. The addition to any of the above monovalent solutions of a slight trace of a divalent salt such as  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ , or  $\text{BaCl}_2$ ,  $\text{CdCl}_2$ , prevents the fall in resistance in the monovalent solution from taking place.

<sup>1</sup> The molecular strengths of the solutions have only been roughly calculated from the conductivity.



Table V.

Lithium chloride Experiment. Emulsion of *B. coli*. Temp. 25° C.  
Cell constant =  $4.22 \times 10^{-1}$ .

1.	Resistance of Ringer and LiCl (0.195 M.) each	...	...	...	26.7 ohms.
2.	Resistance of <i>B. coli</i> emulsion in Ringer's sol	...	...	...	147.0
3.	Resistance due to bacteria, 147 - 26.7	...	...	...	120.3
4.	Resistance of same emulsion in LiCl sol. (0.95 M.) after 25 min.	...	...	...	113.0
5.	Resistance of same emulsion in LiCl sol. (0.95 M.) after 35 min.	...	...	...	100.0
6.	Resistance of same emulsion in LiCl sol. (0.95 M.) after 50 min.	...	...	...	86.0

Table VI.

Rubidium chloride Experiment. Emulsion of *B. coli*. Temp. 25° C.  
Cell constant =  $9.5 \times 10^{-1}$ .

1.	Resistance of Ringer and RbCl	...	...	...	85 ohms.
2.	Resistance of emulsion of <i>B. coli</i> in Ringer	...	...	...	120
3.	Resistance due to bacteria, 120 - 85	...	...	...	35
4.	Resistance in RbCl sol. after 40 min.	...	...	...	105
5.	Resistance in RbCl sol. after 50 min.	...	...	...	100

Table VII.

Hydrochloric acid Experiment. Emulsion of *B. coli*. Temp. 25° C.  
Cell constant =  $9.5 \times 10^{-1}$ .

1.	Resistance of Ringer and HCl (0.049 M.) each	...	...	...	85 ohms.
2.	Resistance of emulsion of <i>B. coli</i> in Ringer	...	...	...	110
3.	Resistance of same emulsion in HCl after 45 min.	...	...	...	128
4.	Resistance of same emulsion in HCl after 65 min.	...	...	...	126

It was possible to transfer the same emulsion from Ringer to NaCl a number of times in succession, and get a fall each time in NaCl and a return to the normal resistance in Ringer. (See Curve II.)

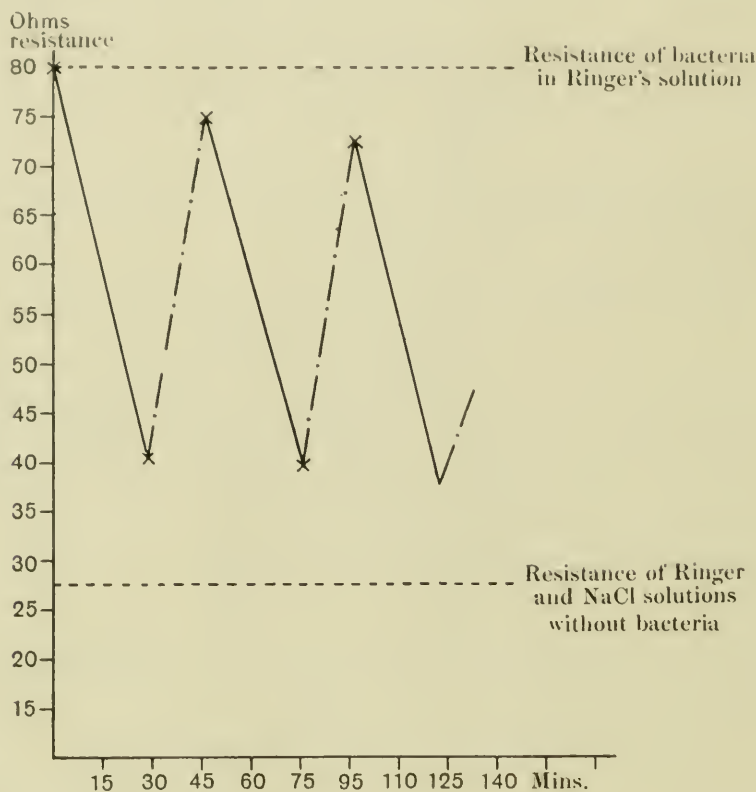
Of the members of the monovalent group, an exception must be made in the case of the H ion. A rapid rise in resistance takes place with all emulsions of bacteria placed in weak solutions of HCl, of the same conductivity as Ringer's solution; as will be seen on consulting the Table and Curve dealing with this experiment.

If, on the other hand, a bacterial emulsion is made up in a solution of some bivalent chloride, such as  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ ,  $\text{BaCl}_2$ <sup>1</sup>, of the same conductivity as Ringer's solution, if the emulsions are sufficiently thick to give fairly high resistances, a slight rise in resistance usually takes place. This is followed after 6 or 9 hours by a slow fall. If, after 12 or 15 hours when this fall is well marked, the emulsion is returned to Ringer's solution, van't Hoff's solution, sea-water, or any balanced solution, it will be found that the emulsion does not regain its normal conductivity, as when a little  $\text{CaCl}_2$  is added to a monovalent salt. There is this difference between the fall of resistance of an emulsion in a monovalent and a divalent salt, that in the latter the fall is irreversible.

<sup>1</sup> In the  $\text{CaCl}_2$  and other bivalent salt solutions the emulsions are always more viscid and less fluid than in the monovalent salt solutions.

Curve II. Experiment showing the antagonistic action of  $\text{CaCl}_2$  in Ringer's solution to the action of pure NaCl solution of the same conductivity as Ringer's solution. Meningococcus emulsion. Temp.  $25^\circ\text{C}$ . Unbroken lines represent the meningococcus in NaCl solution, broken lines—same emulsion in Ringer's sol., the emulsion being transferred three times from NaCl to Ringer's sol. In each instance that the emulsion was placed in NaCl the resistance fell to rise again almost to the normal when transferred back to Ringer.

A similar curve was obtained when a pure  $\text{CaCl}_2$  solution was used of the same conductivity.  $\text{NaCl} = 0.124\text{ M}$ .



If, in place of living, we use an emulsion of dead bacteria for any of the foregoing experiments, none of these changes take place. They are therefore dependent on the cells of the emulsion being alive, as already mentioned; dead bacteria offer no resistance to the passage of the ions of a solution. The gradual fall of resistance which is irreversible in a bivalent chloride is the result of the slow death of the emulsion.

It may be suggested that these effects are to some extent the result of injury to the cell by the electric current or its forcing the ions of the solution through the cells. This cannot be the case, as the action of the monovalent cation is as marked, when the bacteria are placed in these solutions in the absence of any electrical current, as when a current is passing.

In a previous paper (1917) I have demonstrated that the meningococcus is rapidly killed if allowed to remain in 0.85 per cent. NaCl solution for a short time, and that this toxic effect is avoided if a small trace of  $\text{CaCl}_2$  is added to the solution. Thus in the absence of any current we can get the same results as when a current is employed. There is a large amount of evidence to show



that the characteristic action of a monovalent as compared with a bivalent salt in pure solutions is universal for all living cells.

Loeb (1906) has described similar effects of the action of dilute NaCl on the egg of the marine teleost *Fundulus*. The eggs of this fish develop normally in sea-water. If they are put into pure NaCl having the same concentration as that of the sea-water, none of them develop. If, however, a trace of  $\text{CaCl}_2$  is added, as many eggs develop as in ordinary sea-water. The same effect is produced if a little Sr, or Ba, is added in place of the  $\text{CaCl}_2$ . He has also shown (1906) that muscle tissue contracts rhythmically when immersed in pure solutions of salts with a monovalent cat-ion, such as Na, Li, Rb and Cs, but that the addition of a small quantity of a bivalent cat-ion inhibits these contractions.

The interest of the foregoing results consists in that they show that the action of electrolytes on bacteria is similar in all respects to their action on plant and animal cells, as determined in different ways by Loeb and Wastenays (1915), Osterhout (1915), Gray (1916), McClendon (1910), Brooks (1917), and other workers.

In *Laminaria*, Osterhout (1915) finds that with  $\text{CaCl}_2$ , and also with  $\text{BaCl}_2$  and  $\text{SrCl}_2$ , there is invariably a brief temporary rise of resistance when placed in these solutions of the same conductivity as that of the sea-water employed in his experiments. This is followed by a gradual fall. On account of the low resistance used in the present experiments, this preliminary rise of resistance is not so marked. Its presence was frequently demonstrated with  $\text{CaCl}_2$  and  $\text{SrCl}_2$ , as the data and curves of some of the experiments given in this paper plainly show. In the few experiments done with  $\text{BaCl}_2$ , it was not noticed, but it is clear that this salt can obviously antagonise the action of NaCl, and it undoubtedly belongs to the same group, and probably does not differ from them in this respect.

Table VIII.

Magnesium chloride Experiment.

Temp. 25° C. Cell constant =  $4.22 \times 10^{-1}$ .

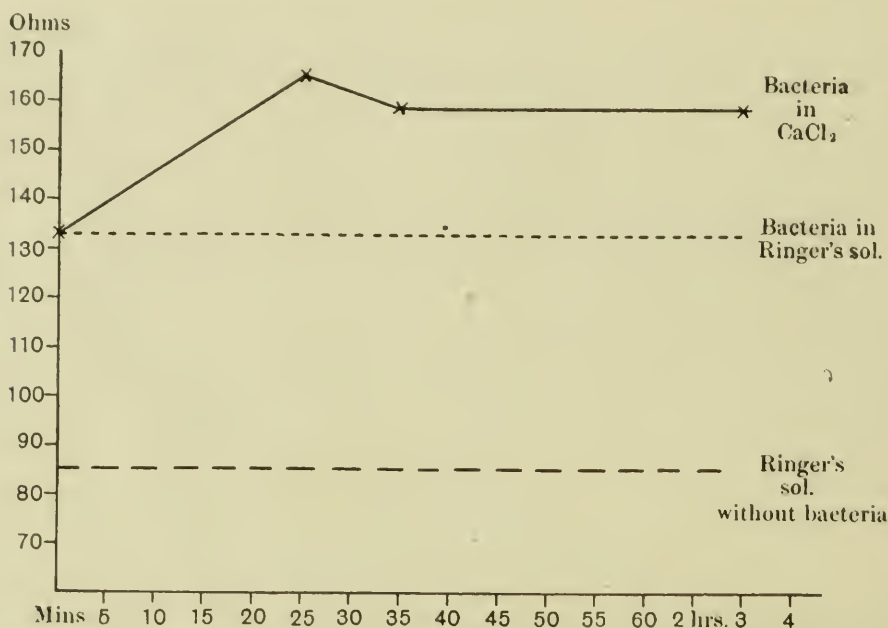
1. Resistance of Ringer and $\text{MgCl}_2$ solutions each ...	...	26.7 ohms.
2. Resistance of <i>B. coli</i> in Ringer's sol. ...	...	108.0
3. Resistance due to bacteria, 108 - 26.7 ...	...	81.3
4. Resistance of same emulsion in $\text{MgCl}_2$ sol. (0.09 M.) after		
10 min. ...	100 ohms resistance	
20 ...	88	
25 ...	80	
35 ...	72	
45 ...	72	

1 drop 40 per cent. formalin added. Resistance fell to 28 ohms.

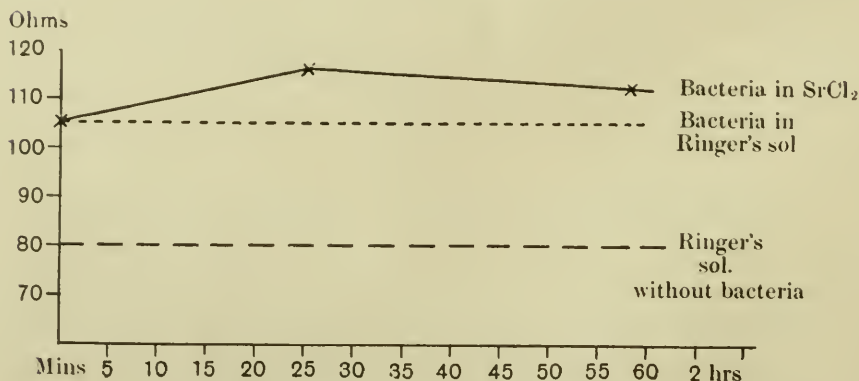
In the light of the present experiments, the time-honoured practice of suspending living bacteria in normal physiological saline (NaCl 0.85 per cent., 29/200 or 0.145 M.) would have little to recommend it, beyond the ease with which most bacteria can be emulsified in such a solution.

As usually employed in practical bacteriology, the action of the NaCl seldom really comes into play, as sufficient  $\text{CaCl}_2$  is always brought over from the culture medium to completely antagonise the NaCl. In Ringer's solution for instance the amount of  $\text{CaCl}_2$  present is only 0.00187 M. Thus in an ordinary bacterial suspension, unless this has been washed once with saline, sufficient  $\text{CaCl}_2$  is present to prevent the specific action of the NaCl. This is doubtless the reason why the injurious action of this salt in ordinary bacteriological manipulation has been so rarely noticed, Flexner (1907) in his classical paper on the meningococcus being one of the few to draw attention to it.

The use of saline solution in practical bacteriological work seems to have originated in a somewhat mistaken opinion of the importance of the rôle of osmotic pressure with regard to bacteria, for which the well-known paper of Fischer (1895), on the plasmolysis, is largely to blame. In distinction to red blood cells, bacteria can easily withstand wide changes in osmotic pressure. In the case of the meningococcus I have shown that this germ can readily survive 24 hours in pure glass distilled water. This is otherwise if the water



Curve III. Calcium chloride Experiment II.  $\text{CaCl}_2 = 0.1 \text{ M}$ .

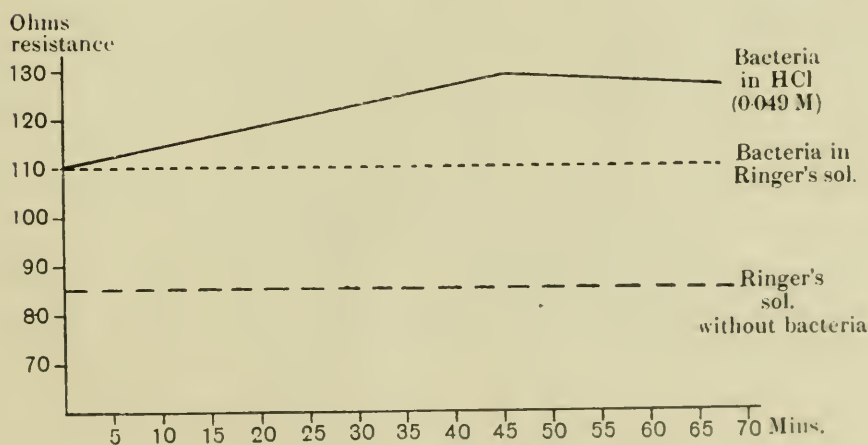
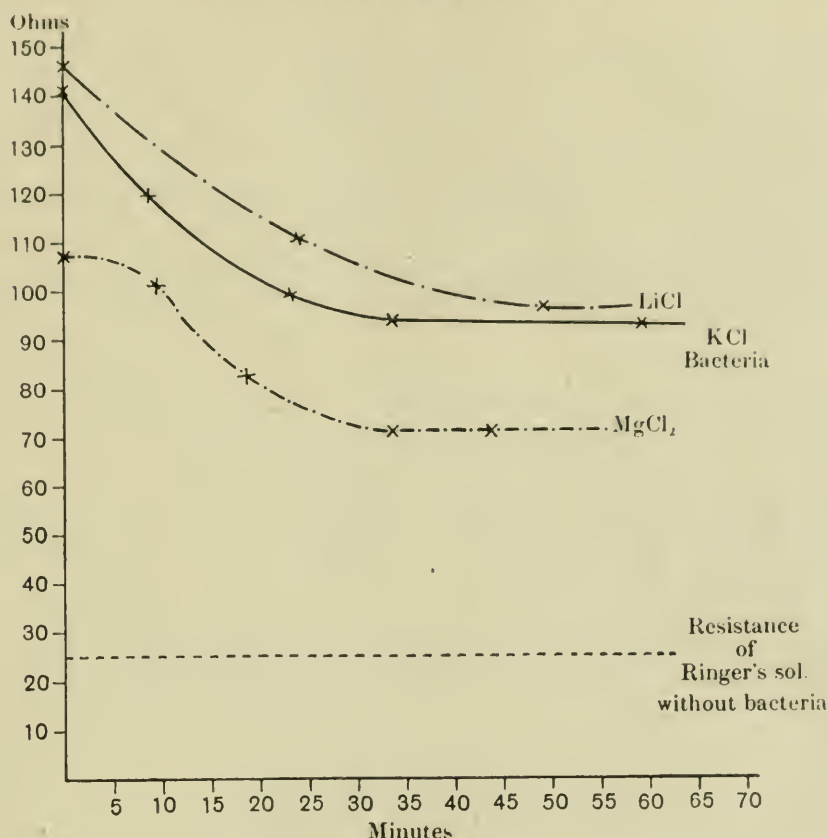


Curve IV. Strontium chloride Experiment.



has been obtained from a metal still. Osterhout (1913) has also drawn attention to the fact that glass distilled water has no toxic action on *Spirogyra*, while metal distilled water has a marked action in this respect.

Curve V, showing the resistance of *B. coli* in Potassium chloride ( $-0.09$  M.) solution.  
 $\text{LiCl} = 0.195$  M.  $\text{MgCl}_2 = 0.09$  M.



Curve VI. Hydrochloric acid Experiment.

In regard to the action of  $\text{MgCl}_2$ , Osterhout (1915) found that a close study of the action of this salt revealed the fact that, although the fall due to its toxic action was very abrupt, resembling at first sight that of  $\text{NaCl}$ , this salt really acts like  $\text{CaCl}_2$ ,  $\text{BaCl}_2$  and  $\text{SrCl}_2$ , in that it produces a short temporary

rise in resistance, followed by an irreversible slow fall in resistance. If this should be the case then the fall in resistance obtained with bacteria should be irreversible. The following experiment would seem to show that this is the case, and that  $MgCl_2$  in its action is to be classed with the other bivalent chlorides as  $BaCl_2$ ,  $SrCl_2$ , and  $MnCl_2$ .

Table IX.

Experiment Demonstrating the Antagonistic Action of  $BaCl_2$  to  $NaCl$ . Temp.  $25^{\circ}C$ .

- (a) To 100 c.c.  $NaCl$  of the same conductivity as Ringer's sol. a few crystals of  $BaCl_2$  were added until the resistance was 20 ohms.
- (b) To 100 c.c. Ringer's sol. distilled water was added till the resistance equalled 20 ohms.
1. Resistance of *B. coli* emulsion in (b) was 95 ohms.
  2. Resistance due to presence of bacteria was 75 ohms.
  3. Resistance of same emulsion of *B. coli* (a) after 15 min. was 95 ohms.: practically no change.

Table X.

Irreversible action of  $MgCl_2$  on bacteria.  
Temp.  $25^{\circ}C$ . Cell constant  $=9.5 \times 10^{-1}$ .

1.	Resistance of Ringer and $MgCl_2$ solutions each ...	...	83 ohms.
2.	Resistance of emulsion of <i>B. coli</i> in Ringer	...	138
3.	Resistance due to bacteria, 138 - 83	...	55
4.	Resistance in $MgCl_2$ sol. after 25 min.	...	96
5.	Resistance of above emulsion plus 5 drops $CaCl_2$ (0.1 M.)	...	88
6.	Resistance of above emulsion washed 3 times in fresh Ringer	...	88

Table XI.

Cadmium chloride Experiment.  
Temp.  $25^{\circ}C$ . Cell constant  $=9.5 \times 10^{-1}$ .

1.	Resistance of Ringer and $CdCl_2$ solutions each ...	...	85 ohms.
2.	Resistance of emulsion of <i>B. coli</i> in Ringer	...	115
3.	Resistance of emulsion of <i>B. coli</i> in $CdCl_2$ solution (0.065 M.)	...	116

Emulsion actively motile at the end of the experiment.

Table XII.

Strontium chloride Experiment.  
Temp.  $25^{\circ}C$ . Cell constant  $=9.5 \times 10^{-1}$ .

1.	Resistance of Ringer and $SrCl_2$	...	...	80 ohms
2.	Resistance of emulsion of <i>B. coli</i> in Ringer's sol.	...	...	105
3.	Resistance of same emulsion in $SrCl_2$ sol. after			
	25 mins.	...	115 ohms.	
	60	...	112	

In view of the results obtained in the foregoing experiments of the action of the monovalent and divalent salts in altering the normal conductivity of the bacterial cell, it is highly important to determine if these changes are correlated in any way with a modification of pathogenic power. To determine this fresh cultures of *B. anthracis* and the pneumococcus were used. The pneumococcus strain was passed through a number of mice before being used.



Emulsions of these bacteria were treated as in the preceding experiments, with NaCl, CaCl<sub>2</sub> and Ringer's solution, and injected into mice and the effects of these solutions on pathogenetic powers observed. In all instances the salt solutions used in these experiments were carefully sterilized before being employed for making up the emulsions of bacteria. The results of these injections are shown in Tables XIV and XV.

Thus a fresh culture of *B. anthracis* was made up in an emulsion in Ringer's solution, its resistance determined, and then transferred to NaCl solution of the same conductivity as the Ringer's solution, being rapidly washed in several changes of NaCl solution to eliminate all trace of the Ringer's solution. It was allowed to remain in the NaCl solution (0.124 M.) for about fifteen minutes, till its resistance had fallen about two-thirds of the way to that of the NaCl solution alone without bacteria, and an estimated dose was then injected into several or more mice. A similar dose of the same emulsion, which had remained in Ringer's solution all the time, was injected into a second lot of mice, of approximately the same weight as those of the first batch. Into a third group of mice a similar dose of the same emulsion was injected in NaCl solution, to which a trace of CaCl<sub>2</sub> had been added. All these emulsions were diluted down in their respective fluids, before being injected, so they rendered these fluids slightly turbid. The results obtained from experiments of this kind were invariably uniform.

It was found that when the resistance had fallen in the NaCl solution, the bacteria failed to kill the mice, or only did so after seven or eight days' time, while those mice that had received the same emulsion in a similar dose in Ringer's solution were sometimes dead within 18 hours and were always dead within 24 or 36 hours. The same applies to the mice that had received the germs in NaCl, to which a little CaCl<sub>2</sub> had been added. (See Tables XIV and XV.)

It would seem that either the majority of the bacteria in the NaCl solution were dead, when they were injected into the animals, or that the action of the NaCl was such as to render them almost harmless. Similar results were obtained when special care was taken to see that the resistance of the germs in the NaCl had not fallen too low. Subcultures made from these emulsions, at the time they were injected, gave a very good growth after 24 hours' incubation, showing that a fair percentage of the bacilli were still alive. There was therefore some evidence for thinking that the NaCl had a detoxicating action on the germs.

It was found when some of these bacilli were exposed in NaCl solution (0.124 M.) to the action of washed leucocytes, they were taken up by these leucocytes immediately, and within a short time few free bacteria remained outside the phagocytes. The same bacteria in Ringer's solution under similar conditions were taken up at a much slower rate by the leucocytes. This result again may be due to the detoxicating action of the NaCl on the bacteria, rendering them more liable to attack by the leucocytes.

If on the other hand we inject the same bacteria in similar doses in  $\text{CaCl}_2$  solution (0.1 M.) they kill the mice in even smaller doses than when they are injected in Ringer's solution. In such a solution the preceding experiments show that a slight rise of resistance takes place. It was found however that the  $\text{CaCl}_2$  solution itself without any bacteria often had an ill effect. In this respect  $\text{CaNO}_3$  seemed more toxic than  $\text{CaCl}_2$ . If two lots of mice were injected, one with a lethal dose of *B. anthracis* in  $\text{CaNO}_3$  and the other with the same germ in  $\text{CaCl}_2$ , the batch that had received the bacteria in  $\text{CaNO}_3$  always were the first to die. In making experiments with  $\text{CaCl}_2$ , control mice were always injected with some of  $\text{CaCl}_2$  alone without bacteria; it was seldom that these showed any ill effects from the dose of Ca employed in the previous injections. It is clear that in the  $\text{CaCl}_2$  solutions no detoxication takes place if the bacteria are injected after standing in this solution for 30 minutes, and possibly a slight increase of toxicity takes place.

### III. DISCUSSION OF THE BEARING OF SOME OF THE RESULTS OF THE FOREGOING EXPERIMENTS ON THE RÔLE OF CERTAIN SALTS IN THE QUESTION OF WOUND INFECTION.

These results have a direct bearing on some recent research on wound infection and gas gangrene.

Bullock and Cramer (1919) find that the organisms of gas gangrene, when emulsified in saline ( $\text{NaCl}$  0.85 per cent. or 0.145 M.) and washed in several changes of this fluid to remove toxins, fail to kill mice and guinea-pigs, while similar doses of broth cultures of these organisms produce a violent gas gangrene which kills the animals in twenty-four hours. In washing the bacteria in several changes of  $\text{NaCl}$  to eliminate all toxins, it will be seen that they are repeating the conditions of the preceding experiments with this salt, where the  $\text{NaCl}$  destroys the normal stability of the cell wall and cytoplasm, which has been shown to be correlated by experiments on animals with a loss of toxicity. They find further that this action can be prevented by adding a little Ca salt, either to the culture in saline when it is being injected, or by injecting the animal a short time after with a large dose of Ca. In this case they are simply antagonising the action of the  $\text{NaCl}$ , and under these conditions, as has been shown in the previous experiments, the conductivity of the bacterial cell returns to its normal condition, and the germs regain their toxic power.

Unfortunately, to determine if the action of the Ca salts is on the bacteria or the tissues of the animal into which the bacteria have been injected, they incubated their cultures for three hours in weak  $\text{CaCl}_2$ ; "the suspension was then centrifuged, and the bacteria after washing with saline suspended in  $\text{NaCl}$ , and the suspension then injected into mice" (p. 521). As would be expected they find that after this treatment the germs are still non-toxic. They conclude from this that the action of the  $\text{CaCl}_2$  is not on the bacteria, and therefore must be on the tissues of the animal.



To this action they give the name of "kataphylaxis." It would seem, however, that the real point in question has been overlooked and this is the action of the NaCl on the bacteria. In testing the action of the  $\text{CaCl}_2$  on the germs, they make the mistake of washing this away with NaCl. In washing once in NaCl and resuspending the bacteria in this solution for injection, they have removed all trace of  $\text{CaCl}_2$ , and the unantagonised action of the NaCl again comes into action. The germs are now in the same state as they were previous to treatment with  $\text{CaCl}_2$ , and are unable to kill the animals.

No doubt the injection of the NaCl or the  $\text{CaCl}_2$  may lead to a certain amount of injury to the tissues at the local point of injection, due to the action of these salts on the tissue cells, but this action is bound to be as great on the bacteria, unless we assume there is a selective action on the tissue cells. Where the  $\text{CaCl}_2$  is added to the emulsion of bacteria in saline before injection, this cannot take place as a balanced solution results. It is obvious that the action of the salts on the tissues is not the controlling factor, as the result is the same in both cases.

In the light of the previous experiments, Bullock and Cramer's results resolve themselves into a perfectly straightforward problem, involving the action of a bivalent salt in antagonising the destructive action of a monovalent one, on the normal stability of the bacterial cell. It is interesting to note, that they have been able to demonstrate the stabilising action of another bivalent salt, Sr; as this salt in the form of  $\text{SrCl}_2$  has about the same effect on colloidal aggregation as  $\text{CaCl}_2$ , this is what might be expected.  $\text{MgCl}_2$  on the other hand has little action in this respect, and they find it has no rupturing action. In regard to the action of  $\text{CaCl}_2$  on  $\text{MgCl}_2$ , this has also been pointed out by several other observers. Loeb (1906) for instance has called attention to the action of  $\text{MgCl}_2$  in modifying the action of  $\text{CaCl}_2$  on the muscular contraction of the disc of the medusa *Polyorchis*, and has pointed out that this is probably not one of real antagonism. The action of Na citrate in destroying the rupturing action of  $\text{CaCl}_2$  on the tissues is probably a purely chemical reaction in which Ca citrate is formed instead of Na citrate, NaCl also being formed.

An experiment was devised (Table XIII) to determine if Na citrate on being added to  $\text{CaCl}_2$  would prevent this salt from antagonising the action of the NaCl. A solution of three parts NaCl and one part Na citrate was mixed, and to this a trace of 0.125 M.  $\text{CaCl}_2$  was added. The solution was then adjusted by the addition of a little distilled water to have the same conductivity as Ringer's solution. A thick emulsion of *B. coli* was then washed in this solution several times and finally allowed to stand in it for an hour. It will be seen on consulting the following table, giving the details of this experiment, that at the end of this period the resistance of this emulsion had dropped very appreciably. It is obvious that the  $\text{CaCl}_2$  has failed to prevent the NaCl from lowering the resistance of the emulsion. Bullock and Cramer when they use these salts together are undoubtedly repeating the conditions of this experiment.

Table XIII.

Experiment to show the action of Na citrate on CaCl<sub>2</sub>.

Solutions made up as follows:

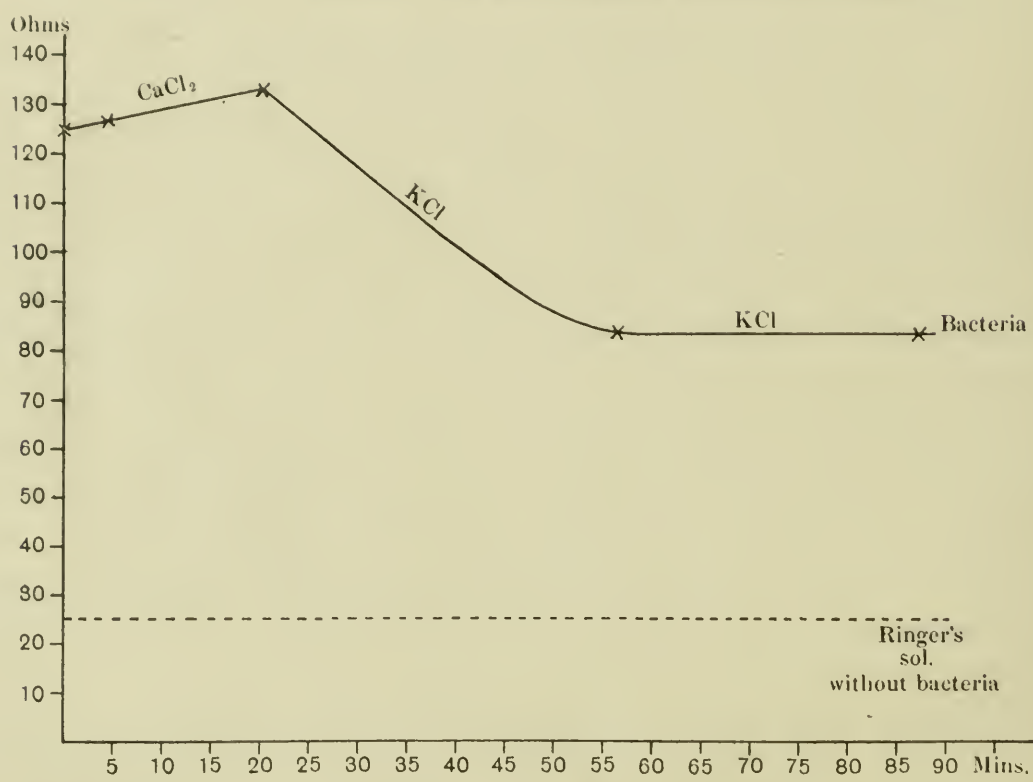
- 3 parts NaCl, 0.124 M. of the same conductivity as Ringer.
- 1 part Na citrate of the same conductivity as Ringer.

To 100 c.c. of above solution 1 c.c. of 0.125 M. CaCl<sub>2</sub> was added and shaken. Distilled water was then added till the conductivity was the same as that of Ringer's solution.

1.	Resistance of Ringer's sol.	...	...	...	...	...	85 ohms.
2.	Resistance of NaCl plus Na citrate plus CaCl <sub>2</sub>	...	...	...	...	...	85
3.	Resistance of <i>B. coli</i> emulsion in Ringer	...	...	...	...	...	131
4.	Resistance due to bacteria, 131 - 85	...	...	...	...	...	46
5.	Resistance in NaCl plus Na citrate plus CaCl <sub>2</sub> after 1 hr.	...	...	...	...	...	90.6
6.	Drop in resistance in above solution after 1 hr.	...	...	...	...	...	40.6

The CaCl<sub>2</sub> in the above solution has not antagonised the action of the NaCl on account of the presence of the Na citrate.

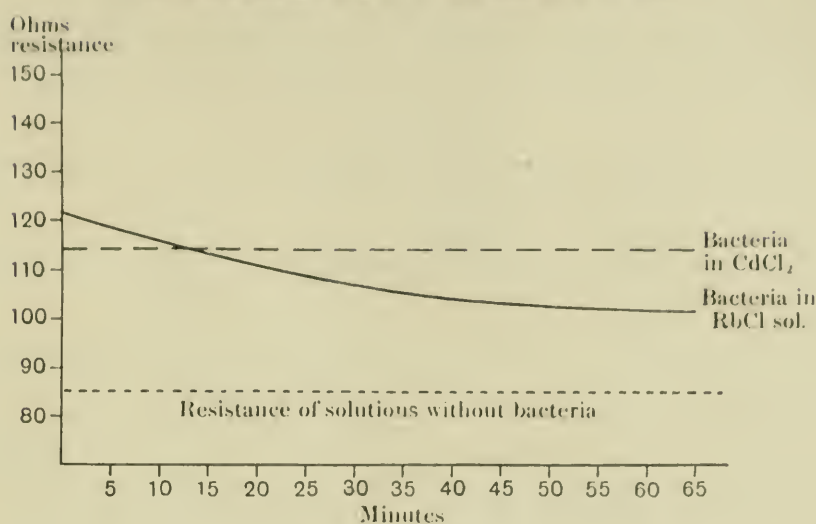
Curve VII. Calcium chloride and Potassium chloride Experiment.



In a previous paper Cramer (1918) makes some observations on the action of NaCl and CaCl<sub>2</sub> in affecting the growth of cancer cells. He found that if equal volumes (about 12 c.c.) of sterile M/7.5 solutions of NaCl and CaCl<sub>2</sub> in tap water were placed separately in two test-tubes, and 0.5 c.c. of a fresh emulsion of cancer cells were added to each and shaken up for an hour or so, the emulsion in CaCl<sub>2</sub> on injection into mice failed to grow as well as the control emulsions. The NaCl on the other hand grew at about the same rate as the controls, and the treatment with this salt had no effect in retarding the growth of the cancer cells.



Curve VIII. Rubidium chloride and Cadmium chloride Experiment. Temp. 25° C.  
Cell constant =  $9.5 \times 10^{-1}$ . Emulsion of *B. coli*.



Cramer does not state if the cancer cell emulsions were first washed several times in distilled water or several changes of NaCl before being finally allowed to remain in the NaCl, to remove all possibility of any Ca being brought over with the emulsion. It is well known that some cancerous growths may contain very considerable quantities of Ca. This amount of Ca combined with that possibly present in the tap water employed might very seriously interfere with the action of the NaCl, especially as the volume of NaCl used was rather small.

All these conditions may have been duly guarded against in this work, but no information is given to this effect in the description of the experiments.

In this paper as well as in the previous one it does not seem to have been realised what a small quantity of CaCl<sub>2</sub> is required to antagonise the action of NaCl. The ratio of these salts for instance in van't Hoff's solution being 2.3 to 100, and in the Ringer's solution used in the previous experiments the strength of the CaCl<sub>2</sub> is only 0.00187 M. as already mentioned.

The ions of one salt are said to antagonise another, when both are simultaneously present in a solution, and each prevents the other from entering the cell or exerting its specific action on the stability of the cell-wall and cytoplasm. Cramer therefore, in placing an emulsion of cancer tissue in a solution of M/7.5 CaCl<sub>2</sub> for a certain time and at the end of this period decanting the cells and resuspending them in M/7.5 NaCl solution for some time, is not strictly speaking dealing with a condition of true antagonism<sup>1</sup>. It is more or less a chance that under these circumstances antagonism takes place.

It is a question, moreover, if cancer tissue can be broken up and emulsified sufficiently to allow the NaCl to act on the cells. I have pointed out in my paper on the action of NaCl on the meningococcus that it fails to kill this

<sup>1</sup> These solutions are not isosmotic as stated in this paper.

Table XIV.

Experiments with *B. anthracis* on mice, a fresh 24 hr. culture being employed. The bacilli, after their conductivity had been determined in thick emulsions, were diluted down in each case to a strength approximately equal to about one million germs to the cubic centimeter; in this dilution they were injected in varying doses. 0 = animal alive and well, + = animal dead, h = hrs., d = days. All salt solutions used for these experiments had been carefully sterilized.

*B. anthracis*<sup>1</sup>.

No. mouse	Weight mouse	Dose and character of solution injected			Result	Remarks
1	18 grams.	0.25 c.c. in 0.124 M. NaCl sol.			+ 24 h	Small animal
2	22.5 "	"	"	"	0 4 d	
3	30 "	"	"	"	0 "	Slightly ill after 48 hrs.
4	27 "	"	"	"	0 "	
5	25 "	0.5	"	"	0 "	
6	28 "	"	"	"	+ 3 d	No signs of illness till 3rd day
7	31 "	"	"	"	0 4 d	
1	26 "	0.25 c.c. in Ringer's sol.			+ 24 h	Some of these had been dead some time when examined at end of 24 hrs.
2	20 "	"	"	"	+ "	
3	29 "	"	"	"	+ "	
4	32 "	0.5	"	"	+ "	
5	27 "	"	"	"	+ "	
1	20 "	0.25 c.c. in CaCl <sub>2</sub> solution			+ 24 h	
2	26.5 "	"	"	"	+ "	
3	21.5 "	"	"	"	+ "	
4	26 "	0.5	"	"	+ "	
1	28 "	0.5 c.c. CaCl <sub>2</sub> , without bacteria			0 24 h	No signs of illness
2	30 "	"	"	"	0 "	
1	19 "	0.5 c.c. NaCl + CaCl <sub>2</sub> solution *			+ 24 h	
2	29 "	"	"	"	+ 48 h	
3	27 "	"	"	"	+ 24 h	

\* Composed of 200 c.c. 0.85 % sterile NaCl + 0.04 c.c. M/1 CaCl<sub>2</sub> and adjusted to have the same conductivity as Ringer's solution.

Table XV.

Experiments with pneumococcus on mice. The pneumococcus strain had been passed through six mice previous to its use in this experiment. All emulsions were diluted down to approximately the same strength before being injected into the animals. 0 = animals alive and well, + = animal dead, h = hours, d = days.

*Pneumococcus*.

No. mouse	Weight mouse	Dose and character of solution injected			Result	Remarks
1	25 grams.	0.25 c.c. in Ringer's solution			+ 24 h	
2	30 "	"	"	"	+ "	
3	21 "	0.5	"	"	+ "	
4	23 "	"	"	"	+ 48 h	
1	31 "	0.25 c.c. in 0.124 M. NaCl sol.			0 24 h	Dead 3rd day
2	19 "	"	"	"	0 "	
3	25 "	"	"	"	0 "	
4	22 "	"	"	"	0 "	
5	28 "	0.5	"	"	0 "	

<sup>1</sup> The groups of animals to which the three consecutive tables relate were in each case injected with the same bacterial emulsion.



Table XVI.

*B. anthracis.*

No. mouse	Weight mouse	Dose and character of solution injected			Result
1	26.5 grams.	0.25 c.c.	Ringer's solution		+ 24 h
2	31 "	"	"	"	+ "
3	29	"	"	"	+ 48 h
1	22 "	0.25 c.c. in 0.124 M.	NaCl sol.		0 3 d
2	27 "	"	"	"	0 "
3	29 "	"	"	"	0 "
1	30 "	0.25 c.c. in 0.124 M. NaCl +	trace Ca nitrate		+ 24 h
2	25 "	0.25 c.c. in 0.124 M. NaCl +	trace Ca nitrate		+ "

germ if any clumping of the cocci takes place. On subsequent incubation, the bacteria in these clumps grow and give rise to isolated colonies; the NaCl seem unable to affect the cocci in the interior of the clumps. In order that the NaCl may kill all the bacteria, it is necessary to employ emulsions entirely free from clumps or masses of bacteria. I fail to see how cancer tissue can be emulsified so thoroughly that all the cells are separated, and the NaCl given free scope to act.

I believe this reason, as well as the fact that no special precautions were taken to see that no Ca was introduced with the cancer tissue cells in these experiments, accounts for the failure to get the action of the NaCl. In these experiments the real action of the NaCl on the cancer cells has not been properly demonstrated. There is reason to think that if they were repeated with due attention to these points, the action of this salt on the cancer cell would be the same as on all other animal and plant cells. That sufficiently long exposure to pure solutions of NaCl in a concentration of 0.145 M., say two or three hours, would result in the death of these cells.

Thus the failure to distinguish the characteristic action of NaCl on living cells, and its highly reversible nature, renders the results of Cramer, Bullock and Cramer open to a different interpretation from that given by them.

No evidence has been advanced by Bullock and Cramer to show that the action of the NaCl on the bacteria is not the decisive factor in the matter. If a similar effect could be produced by the injection of the bacteria in distilled water, there might be some ground for believing that the action of the Ca was on the tissues. If on the other hand it can only be obtained by the injection of bacterial suspensions that have been washed several times in NaCl, in the light of the present conductivity experiments the explanation is obvious.

We come finally to the question as to what do these changes in the conductivity of bacterial emulsions signify? Osterhout, in his experiments on plant tissues, believes that they give us definite values for the permeability of the tissues to the ions. This view has been openly challenged by Stiles and

Jørgensen (1918), who point out that this is to a certain extent a pure assumption.

In placing living cells in pure salt solutions, no matter how weak, it is doubtful if we are investigating permeability at all, but simply the destructive action of the salt on the cell, as the result of which a certain amount of ex-osmosis or endosmosis takes place. Neither Osterhout's experiments, nor those of Stiles and Jørgensen, or Stiles and Kidd (1919), give us any information as to how absorption takes place from a balanced solution. It would seem that the problem is one more for the protein chemist than for the experimental physiologist.

Sørensen (1917), in his extensive studies on the physical properties of proteins, has shown that the capacity of egg-albumen to combine with acids and bases, at a certain hydrogen ion concentration, is a function of the amount of ammonium sulphate present, and is greater as the latter increases.

It is possible on a basis of a measurement of the number of hydrogen ions present in a solution of egg-albumen, having a known composition and containing ammonium sulphate, to estimate the total content of surplus acid, and to determine by formula its approximate distribution between the two phases of the egg-albumen solution.

It will perhaps be from investigations of a similar character, on the action of the ions of Na, K, and Ca, on the physical properties of the proteins and lipoids, that will show us how these ions stabilise the cell membrane and cytoplasm, for it is clear that in the presence of these ions the semi-permeability of the cell-wall remains constant.

Schryver (1913), in a series of researches on the formation of gels from cholate solutions, has shown that Na cholate solutions set to a gel when heated in the presence of Ca. This gel formation is readily inhibited by the presence of relatively small quantities of NaCl. In further experiments, he showed that cholate gels are eroded when immersed in solutions of NaCl and other chlorides, and that this erosive action can be antagonised by very small amounts of  $\text{CaCl}_2$ . Quantitatively, the chloride solutions in their erosive action differ considerably from one another, the order of their action being as follows commencing with the greatest, LiCl, NaCl,  $\text{MgCl}_2$  and KCl<sup>1</sup>. It will be seen that we are dealing in these experiments with similar conditions to those demonstrated by the previous conductivity experiments on living bacteria.

In order to see if the solutions found by Schryver to produce erosion of a cholate gel would also produce a change in the conductivity of bacteria emulsions, certain experiments were made; they all gave negative results, however, where chloroform and chloral hydrate<sup>2</sup> were used, two substances that gave the highest cholate gel-destroying capacity in Schryver's work.

<sup>1</sup> The effects of the action of these salts on the permeability of vegetable tissues follows the same order, as determined by Stiles and Jørgensen (1915). In the case of bacteria no attempt was made to determine the order of their action, as the resistances employed were too low to allow of any relative comparison.

<sup>2</sup> These experiments need repetition.



## IV. SUMMARY.

The action of univalent and bivalent salts on bacteria in affecting the conductivity of thick emulsions of the meningococcus and *B. coli* demonstrates the important fact, that they alter the conductivity of these germs in the living condition, in a very definite manner. In this alteration, the predominant part is played by the cat-ion.

All monovalent cat-ions, with the exception of the H-ion, such as those of Na, K, Li, Rb, produce a rapid increase in conductivity or a fall in resistance. In its early stages this increase in conductivity is readily reversible in these solutions. If allowed to follow its due course, however, it leads finally to death in about two hours, when the bacterial cells no longer offer any resistance to the passage of the ions. The conductivity of the emulsion then becomes that of the fluid in which the emulsion has been suspended.

Bivalent cat-ions and the H-ion, on the other hand, at first produce a slight fall in conductivity or an increase in resistance, followed, secondly, by an irreversible increase in conductivity which is slow and gradual resulting finally in death after 48 or more hours. This is shown by the cat-ions of Ca, Sr, Ba, and Cd.

In a balanced solution such as sea-water, blood plasma, Ringer's solution, van't Hoff's solution, where a certain quantity of  $\text{CaCl}_2$  antagonises a larger amount of NaCl and KCl, the conductivity of bacterial emulsions undergoes no change, but remains constant.

Dead in distinction to living bacteria offer little resistance to the passage of ions of a solution. It would seem the relatively high resistance of the bacterial cell is due to some condition present in the living and absent in the dead state.

It has been shown by experiments on animals that the condition of increased conductivity of the cell is coupled with a loss of virulence in the case of some pathogenic bacteria. It is not clear from these experiments if this is due to the actual death of the germs in a monovalent salt solution, or to a detoxicating action of these solutions on the germs. It would seem there is some evidence for believing that the latter takes place, as the lost lethal power returns in the presence of a small trace of Ca. In bivalent salt solutions no loss of toxicity takes place. It has been pointed out that the action of monovalent and bivalent salts on bacteria offers a new explanation of certain experiments with gas gangrene organisms, where it is at present considered that the action of the salts are on the tissues of the animal and not on the bacteria.

## REFERENCES.

- BROOKS, C. S. (1917). *Bot. Gaz.* LXIV. 306.
- BUGARSKY and TANGL (1897). *Zeitschr. f. Phys.* II. 297.
- BULLOCK and CRAMER (1919). *Proc. Roy. Soc. Ser. b*, xc. 513.
- CRAMER, W. (1918). *Biochem. Jr.* XII. 210.
- DANYSZ (1900). *Ann. Inst. Past.* XIV. 641.
- FISCHER, A. (1895). *Jahrb. d. Botanik*, XXVII. 1.
- FLEXNER, S. (1907). *Jr. Exp. Med.* IX. 105.
- GIRARD, P. (1910). *Jr. Physiol. Path. gén.* XII. 471.
- (1919 a). *Compt. Rend.* CLXVIII. 1335.
- (1919 b). *Ibid.* CLXIX. 94.
- GORDON, M. H. (1918). *Brit. Med. Jr.* 1918, 110.
- GRAY, J. (1916). *Phil. Trans. Roy. Soc. Ser. b*, CCVII. 481.
- HÖBER, R. (1913). *Archiv ges. Phys.* CL. 15.
- LOEB, J. (1906). *Dynamics of Living Matter*, New York.
- LOEB and WASTENAYS (1915). *Jr. Biochem.* XXI.
- MCCLENDON, J. F. (1910). *Am. Jr. Phys.* XXVII. 270.
- MINES, G. R. (1911). *Jr. Phys.* XLII. 309.
- OKER-BLOM (1900). *Pflüg. Archiv*, LXXIX. 510.
- OSTERHOUT, W. J. V. (1913). *Bot. Gaz.* LV. 446.
- (1915). *Ibid.* LIX. 315.
- (1918). *Jr. Biochem.* XXXVI. 557.
- PERRIN, J. (1904). *Jr. Chimie Phys.* II. 601.
- PORGES (1905). *Wien. klin. Wochenschr.* 1905, 691.
- RÓTH, W. (1897). *Elektrische Leitfähigkeit thierischer Flüssigkeiten.*
- SCHRYVER, S. B. (1913). *Proc. Roy. Soc. Ser. b*, LXXXVI. 460.
- SHEARER, C. (1917). *Ibid.* Ser. b, LXXXIX. 440.
- SØRENSEN, S. P. L. (1917). *Travaux de Labt. Carlsberg*, XII. 1.
- STEWART, G. N. (1899). *Jr. Phys.* XXIV. 356.
- (1910). *Jr. Pharm. Therap.* I. 49.
- STILES and JØRGENSEN (1915). *Ann. Bot.* XXIX. 349 and 610.
- (1918). *Bot. Gaz.* LXV. 526.
- STILES and KIDD (1919). *Proc. Roy. Soc. Ser. b*, xc. 448.
- WOELFEL, A. (1908). *Biochem. Jr.* III. 146.



## THE CULTIVATION OF AEROBIC BACTERIA FROM SINGLE CELLS.

By EDWARD C. HORT, F.R.C.P. (EDIN.).

(With 1 Text-figure.)

IN order to obtain cultures of aerobic bacteria from single cells it is hardly necessary to insist that a method of isolation should be employed which can be relied on to ensure that one organism, and one only, is the starting point for inoculation of the first subculture. Unfortunately, for technical reasons which will be explained, no such method is at present available.

The object of the present note is to show why the present methods of isolation are unsatisfactory, and at the same time to describe a simple and convenient method on which reliance can be placed. The methods at present in use include the fragmented glass method, the Indian ink method, the squared coverslip method, the capillary tube method, the Barber method, and the droplet method, of which the Malone method is a modification. It is assumed that the principles underlying the use of these methods are sufficiently well known to preclude the necessity for a detailed description of each. The fallacies and drawbacks incident to their employment will therefore mainly be dealt with, though a certain amount of description of the less well-known methods is unavoidable.

### THE FRAGMENTED GLASS METHOD.

By this method of isolation, a description of which I am unable to find, an attempt is made to superimpose on selected single cells, scattered over agar or gelatin, small fragments of thin glass in the hope that successful inoculation of a suitable medium, such as broth, will result by placing a selected fragment with its adherent organism in such medium. The disadvantages of the method are as follows:

1. Only dry lenses can be employed.
2. The poor visibility of organisms lying under the prismatic edges of the fragments of glass often makes it impossible to be certain that only one organism is present under the fragment of glass selected.
3. When picking up the selected fragment it is difficult to be certain that organisms lying close to the fragment are not also unwittingly picked up by slight displacement of the fragment during the process of removal.

4. Once the selected fragment has been picked up it is difficult, or impossible, satisfactorily to re-examine it with a view to determining the presence or absence of organisms not previously noticed.

5. As usually employed no control observations of growth from single cell to single colony can be carried out to determine the presence or absence of more than one organism, because the selected fragment is at once placed in broth or other suitable liquid medium. It is of course possible to select a fragment of glass which appears to be lying on one organism only, to incubate the culture until colony formation is well established, and to examine again before removal. In practice, however, disadvantages 2 and 3 come into operation, and invalidate this method of control. Apart from disadvantages 1 to 5, which together prohibit the use of the fragmented glass method for scientific work, there are two minor drawbacks to the method. The first of these is the mechanical difficulty of superimposing small fragments of glass over selected organisms, there being often about 50 per cent. of failures even after considerable practice. And the second lies in the fact that frequently removal of the selected fragment fails to remove also the organism desired.

#### THE SQUARED COVERSIP METHOD.

By this method, described by Hewlett, a large coverslip is employed on which are etched several numbered squares. On the coverslip is poured a thin layer of agar, or gelatin-agar, a minute quantity of a liquid culture of the desired dilution being spread with a glass rod over the agar when set. The coverslip is then inverted over a hollow moist cell, and examined with a dry lens. The position of an individual organism in a given square is then noted, and the culture is incubated. When colony formation is sufficiently marked to be visible to the naked eye the selected colony is removed, and dealt with in the ordinary way. In theory this method sounds promising, because it would appear to allow of control observations being made during development from single cell to single colony, and so of ensuring that one organism, and one only, is the starting point of the desired culture. In practice, however, the method is of little or no value for the isolation of bacteria, especially when small, because the visibility of organisms on an inverted culture is extremely poor, even under optimum conditions of illumination. It is therefore impossible to be certain that only one organism is present in a given square, and that a colony which appears to be derived from one cell only is not in reality derived from two or more coalescing colonies from two or more adjacent cells. And the necessity for waiting until colony formation is sufficiently far advanced to allow of removal under the naked eye greatly increases this danger.



## THE INDIAN INK METHOD OF BURRI.

For accurate work this method, described in detail by Besson, is not satisfactory, owing to the tendency to concealment of minute organisms by the pigment, although theoretically they should stand out in bold relief even when near to the vanishing point of vision. Numerous experiments with Congo-red, and other pigments, have convinced me that these suffer from the same defects as does Indian Ink.

## THE CAPILLARY TUBE METHOD.

In 1916 I experimented for several weeks with round, and with flat, capillary glass tubes filled with dilute broth or gelatin cultures. The best results were obtained with extremely fine flexible strands of tubular glass, attached in short lengths to microscope slides with plasticine, the desired section containing the selected organism being excised with a sterile knife, and dropped into broth. Prolonged examination of these sections at a temperature which precluded multiplication showed however that even under optimum conditions of illumination the method is a treacherous one, and it was therefore abandoned for the isolation of aerobic organisms, though the fact that it is possible to examine these fine tubular threads of glass with an oil-immersion lens was a strong temptation to continue the experiments. A further drawback to the method for aerobic organisms, unless freely motile, is the difficulty of ensuring passage of the contained organism into the surrounding broth, multiplication within the tube apparently not taking place. For anaerobic organisms, as recorded in 1918 by Holker, the method has obvious advantages, though the optical difficulties of ensuring the presence of single organisms still remain.

## BARBER'S METHOD.

By this method an attempt is made to isolate single cells from liquid cultures with the aid of minute pipettes held in a mechanical finger operated by an ingenious adjustment device. This method is unreliable because

(a) Once the pipette has been removed re-examination to determine whether more than one organism is or is not present is impossible.

(b) Control observations of development from single cell to single colony cannot be carried out owing to the exclusive use of a liquid medium in the early stages.

(c) Of the optical difficulties attendant on the examination of droplets (*vide* "the droplet method").

## THE DROPLET METHOD.

In theory this method is a good one, because it allows of the use of an oil-immersion lens. It may be used in one of three ways:

1. A sharp pointed spud of hard wood, sterilized by immersion in 40 per cent. formalin, and dried just before use in sterile wool, is dipped

into a broth culture of the desired dilution. Momentary contact is then made between the point of the spud and the centre of a minute ring cut on a sterile coverslip with a revolving diamond. The inoculated coverslip is then inverted onto the upper edge (moistened with Canada balsam) of a glass collar cemented to a microscope slide, and is examined with a dry lens. The minute droplet is easily recognized in the centre of the etched ring, and provided that its diameter is not greater than that of the microscopic field it can be thoroughly examined in a few seconds with the dry lens, and in a few minutes with an oil-immersion lens. A series of rings, each with its own droplet, can be examined in turn on each coverslip till one droplet is found, apparently containing only one organism. The coverslip is then removed and placed on its back in a sterile Petri dish. A small drop of broth is then delivered into the centre of the ring containing the selected organism, and is recovered with a fine sterile pipette, delivery being finally made into a tube of broth.

2. Delivery is effected by depositing minute droplets with a hair pipette attached with plasticine to a pipette of larger bore provided with a rubber teat, accurate delivery being effected by slight compression of the rubber teat at the exact moment of contact of the tip of the pipette with the centre of each etched ring. As in droplet method I, to ensure rapid examination, the diameter of the drop should not exceed that of the microscopic field afforded by the dry lens employed in the preliminary search.

3. Delivery is effected, on an inverted coverslip in a closed glass cell, of a series of minute droplets from a fine pipette by an ingenious arrangement devised by Malone, using two microscope stands for the purpose. Each droplet is examined in turn, and the selected droplet is finally collected with a fresh pipette, using the same mechanical device for collection as for distribution.

In practice the droplet method, whether delivery be effected by wooden spuds, by the direct pipette, or by the inverted pipette of Malone, is disappointing. This is because the minute size of the drop which must be employed to avoid grave error often involves a fatal retraction of the periphery of the drop, with the danger of leaving stranded in the shrinkage area—even in a moist cell—organisms which rapidly fade from view, but which nevertheless, as experiment shows, are not necessarily dead. This retraction difficulty can be to some extent avoided by using a 20 per cent. solution of glycerin in water as the bacterial vehicle, though the percentage of successful cultivations is in practice small owing to the lethal effect of the necessarily high concentration of glycerin. Even apart however from the retraction difficulty the visibility of bacteria in minute hanging droplets is always poor, and makes it impossible to be absolutely certain that one organism, and one only, is present in the droplet it is desired to use as inoculum. And finally, as in the



case of other methods involving the use of liquid media throughout, it is not possible with any modification of the droplet method to carry out control observations of development from single cell to single colony.

We learn, then, from this brief review of the methods of isolation at present available that for the reason just stated

1. Control observations from single cell to single colony are absolutely essential because of the optical difficulties which attend all known methods, making it impossible to rely upon any results from single observations.

2. Any method which aims at isolation from liquid media is inadmissible. This at once excludes Barber's method, all the droplet methods and the capillary tube methods.

3. For the same reason the fragmented glass method and the Indian Ink method must also be rejected.

We are thus left with only the squared coverslip method which has greater optical disadvantages than any other method, disadvantages which are inseparable from the use of an inverted medium inoculated on its distal surface.

It would seem therefore that the only chance of finding a reliable method of isolation is to employ direct examination of organisms on a solid medium, and to insist on a series of control observations in order to be certain that in the development from cell to colony one cell, and one cell only, is originally present.

At a time when it was believed that bacilli or cocci can only arise from pre-existent bacilli or cocci by equal binary fission the necessity, especially in the case of the larger organisms, for control observations during development from single cell to single colony was—naturally enough—not apparent. Now, however, that it is known that, for example, minute gonidial forms extruded from the mother-cell are often themselves highly fertile it is clear that complete control observations are essential in order to ensure that the unsuspected presence of these minute forms does not give rise to serious error.

Of the two methods now to be described one is suitable for oil-immersion work, and the second for dry lens work only.

#### FOR OIL-IMMERSION LENS.

A series of sterile coverslips is prepared, each with a small ring etched on one of its surfaces. At the same time is also prepared a dozen or more clean sterile microscope slides, over each of which is poured under cover of a Petri dish filtered peptone-agar in a thin layer. A dilute culture is now prepared, and in turn each coverslip is inoculated in the centre of the etched ring with the minutest possible droplet of the culture. Each inoculated coverslip is now placed face downwards on each agar slide, care being taken to ensure direct application of slip to agar without sliding of the former over the latter.

Great care must also be taken to ensure that the droplet of inoculum is sufficiently small not to run outside the ring when firm pressure is exerted over the slip when *in situ*. With a little practice this accident, which is of course fatal to the experiment, can be avoided, it being quite possible to effect delivery of a droplet so small in diameter as to be hardly visible to the naked eye, and well within the field of vision allowed by a dry lens of  $\frac{1}{6}$ -in., and the appropriate ocular. After careful search with the dry lens an oil-immersion lens is substituted, and the whole area within the etched circle is thoroughly examined. If only one organism can be found the immersion oil is carefully removed, and the slide is incubated at 37° C. for four to six hours, being examined at intervals of 20 minutes throughout this period.

When the observer is perfectly satisfied that the colony now in process of formation has started from the original cell, careful drawings being made throughout, and that only that particular cell was originally present within the etched ring, the slide is now replaced in the incubator. Twelve to eighteen hours after inoculation the coverslip is removed with sterile forceps, and a platinum loop charged with broth is rubbed over the area of glass enclosed by the etched ring, the second subculture being then carried out in the ordinary way.

This method, using an oil-immersion lens, is tedious on account of the necessity for removing the oil used at each examination, preparatory to each re-incubation. The brilliancy of outline produced by pressure of the coverslip on the agar surface and the increased amount of detail to be made out by using an oil-immersion lens, more than compensate however for this trifling drawback. And if it is desired, as was the case in most of my studies, to obtain careful drawings of warm-stage development of morphological changes in the passage from single cell to single colony it is incomparably the best method, though the relative loss of oxygen necessitated is certainly a drawback.

The accompanying figure illustrates what actually happens in such a case, and demonstrates how clearly the morphological changes can be followed by using this method, though in this particular case the experiment was not undertaken with a view to subsequent identification of the nascent colony shown.



Fig. 1. *B. typhosus*. Record of development of a colony observed between 4.55 and 6.50 p.m. on + 10 agar, from 4 % glucose broth.



## THE PERFORATED PLATE METHOD—FOR DRY LENS ONLY.

For ordinary purposes of isolation of single cells, however, in which all that is required is a series of careful control observations to ensure that a culture has been started from one organism only—without any necessity, that is, for elaborate studies of morphological changes in individual cells as illustrated in the figure—the use of the dry lens alone is sufficient. An ordinary sterile glass slide is covered with a suitable medium, such as filtered peptone-agar or glucose peptone-agar, by pouring the latter at a temperature of as near 100° C. as possible under cover of the top of a sterile Petri dish. A liquid culture in appropriate dilution is then spread over the medium, as soon as set, with a glass rod, and a thin strip of perforated celluloid, or other suitable material, is placed over the slide. Sterile coverslips are now placed lightly over the celluloid, and the slide is placed within a sterile Petri dish, and is incubated at 28° C. for from 1 to 1½ hours. The object of placing coverslips over the celluloid is firstly to protect the culture medium from dust during subsequent examinations, and, secondly, to convert each of the tiny cells into a moist chamber, aqueous vapour arising from the surface of the moist medium becoming recondensed on the inverted coverslip.

At the end of 1 to 1½ hours the slide is examined cell by cell until the organism it is required to isolate is found, alone in a single cell, or until a cell is found in which a small number of suitable organisms occur so conveniently placed that there is no danger of the resulting colonies impinging on each other. When found the position of the selected cell is noted by the vernier attached to the microscope stage, and is recorded on a diagram with drawings of the organism or organisms it is desired to isolate. The slide is now replaced in its Petri dish and reincubated. It is further examined at short intervals until colony formation from single cells is established, careful search on each examination being made to ensure that organisms previously unrecognizable as bacteria are not now coming into view, and to make, if desired, such careful drawings of alterations in morphology of the organisms originally selected, or of their direct descendants, as is possible with a dry lens. When the colonies have reached a convenient size it will be found that they are still too small for removal under the naked eye, and if they are left until recognizable without the aid of a lens there is always the danger of coalescence of adjacent colonies—if a cell has been selected with more than one organism—and of the experiment being ruined.

If, however, the following simple technique is adopted there is no difficulty in picking off fragments of colonies—the growth of which from individual cells has been watched and recorded *de initio*—whilst yet too small to be detected under the naked eye. The procedure is as follows. The objective in use is replaced by a perforated metal stop, made for the purpose by Angus and Co., Wigmore Street, London, the perforation being conical in shape, with the apex at its lowest point. Into this conical space is dropped a No. 9 solid

steel needle, the point of which, when *in situ*, is blackened in the flame of a match. A minute drop of paraffin wax on a slide has, previously to removal of the objective, been exactly centred on the microscope stage. The blackened needle is now lowered till it touches the wax, the needle receding in its holder at the moment of contact. The position of the blackened point of contact on the white wax is now noted under the replaced objective, and is brought into the centre of the field, if not already there. The whole manoeuvre is repeated a second time in order to be sure that the point of contact is sufficiently approximate. The selected cell, from which the coverslip has been removed, is now examined, and the chosen colony is centred on a hooded stage. The point of the resterilized needle is now touched with broth in a small platinum loop, is brought into contact with the centre of the colony and, after racking up, is again touched with broth in a resterilized platinum loop, from which a tube of broth is inoculated in the ordinary way. Finally the objective is once more restored, and a control observation made that the desired colony has been touched, and no other.

The advantages of the perforated plate method are the following:

1. Extreme simplicity.
2. The expense of the outfit is negligible.
3. It is relatively rapid.
4. The method of cultivation ensures the maximum of oxygen and moisture.
5. Successful cultivation from fragments of the removed colonies takes place in my hands in 100 per cent. of the cases.
6. It affords an excellent control observation throughout against
  - (a) contamination,
  - (b) picking up fragments of a colony from more than one organism.

In conclusion it is necessary to point out that cultivation of bacteria from single cells is, even when employing a good method, a most tedious procedure, involving several hours' close work for each organism isolated, if the results are to be relied on. It cannot be too strongly insisted that all claims to have grown cultures with certainty from single cells must be accepted with reserve unless the whole process has been repeatedly controlled from selection of single cell to transference of the established colony, either in whole or in part.

#### REFERENCES.

- BARBER (1908). The rate of multiplication of *B. coli* at different temperatures. *Journ. Infect. Dis.* v. 379.
- (1911). A technic for the inoculation of bacteria and other substances into living cells. *Ibid.* viii. 348.
- BESSON (1913). *Practical Bacteriology, Microbiology and Serum Therapy*, p. 83.
- BURRI (1909). *Das Tuschenverfahren*. Jena, Gustav Fischer.
- HEWLETT (1918). *Manual of Bacteriology*, 6th ed. p. 535.
- MALONE, R. H. (1918). A simple apparatus for isolating single organisms. *Journ. Pathol. and Bacteriol.* xxii. 222.



## THE REPRODUCTION OF AEROBIC BACTERIA.

BY EDWARD C. HORT, F.R.C.P. (EDIN.).

(With Plates IV—VII.)

### PART I.

UNTIL the year 1916 it was generally believed that the non-sporing bacteria of the lower orders are only capable of reproducing themselves by the simple process of transverse binary fission into two equal parts. This—it was taught—is the beginning and end of their reproductive life. A bacillus, or a coccus, always arises directly from an equally dividing bacillus or coccus, and in no other way whatever. So firmly fixed was this conception of the entire reproductive life of the lower bacteria under all circumstances that any evidence suggestive of the occurrence of other methods of reproduction, such as by budding, or by branching, or by the production of endobodies was apt to be explained away either by alleged contamination of the cultures employed, or by vague references to the phenomena of involution.

In 1916 and 1917, however, and again in 1918, I showed by several series of warm-stage studies of isolated organisms of the enteric group that under certain circumstances the lower bacteria are able to reproduce themselves by the production of fertile branches and buds, and by the endogenous production of gonidial bodies, in addition to the more familiar method by equal binary fission. And I also showed that evidence of this can be found, if looked for, in young cultures in standard media. The cultures I employed were, as I thus proved, pure cultures, so that any suggestion of contamination organisms being present to account for the results obtained was out of the question. And as I had shown that organisms undergoing branching or budding were often fertile organisms, the detached buds and branches themselves giving rise to a new race, it was no longer possible to advance the theory that these were involution forms—in the ordinary sense of the term—since organisms undergoing genuine involutionary changes, recognizable as such, are sterile organisms. The thesis that the lower bacteria can reproduce themselves in more ways than one, when occasion arises, was therefore fully established for the first time, there being no previous record of continuous warm-stage observations to show that isolated organisms belonging to the lower orders of bacteria, and undergoing branching or budding, or endogenous chromatinolysis are capable of perpetuating a new race which is itself fertile, its progeny being culturally, biochemically and serologically identical with the original mother-cell.

Of the experimental observations establishing these facts several were published in 1916 and in 1917. They therefore need not be reproduced here. Of the remaining observations, and the most complete, a few were examined by a War Office Committee (*v. note infra*) specially appointed for the purpose, and are now published—together with further observations—for the first time.

#### INVOLUTION FORMS: GENUINE AND SPURIOUS.

Before presenting the chief facts on which the above statements are based it is necessary to attempt a definition of the vague term involution form, and to show, in passing, how the literature of bacterial morphology is pervaded with misconceptions arising from the lack of any clear understanding of what an involution form, properly so-called, really means.

An involution form of bacterium can only mean a bacterium which is undergoing retrogressive, or perhaps, degenerative changes. It is, strictly speaking, a sterile organism which is not only incapable of maintaining its reproductive activity, but is also incapable of maintaining its integrity of form. It stains irregularly, feebly or not at all: and in the unstained condition it rapidly fades from view as death ensues, and as autolysis proceeds. It becomes misshapen and deformed, quite early—in the case of motile organisms—losing its motility, as well as that perfect symmetry of outline, and diffused receptivity of stain which mark the healthy normal organism. To the trained observer it offers no difficulty of recognition, and is clearly an organism that has failed in the race of life, as a result of profound disturbance—set up by prolonged sojourn in unfavourable environment—of that power of adjustment of osmotic variations on which all cell activities ultimately depend.

There are, however, often to be seen under less unfavourable conditions aberrant types of the lower bacteria, aberrant that is in the possession of buds or branches, or in some other way, which show none of the characteristics of the genuinely involuting organism. They exhibit perfect symmetry of form, they show no irregularity of staining, which is often deep—especially with carbol-fuchsin—and they do not fade from view in the unstained condition. These organisms are none the less often indiscriminately ranged with genuine involution forms, and are often figured as such, when they do not escape detection altogether, in some of the text-books. So general indeed is the tendency to apply the term involution to any form of bacterium departing from the usual that the legend is unblushingly transcribed from book to book. And the result has been that until recently no effort has been made to apply the crucial test of isolating healthy looking bacteria of the lower orders undergoing branching or budding, and of then determining their ability or inability to produce a new race culturally, biochemically and serologically identical with normal organisms. If an aberrant type of bacterium—neither bacillary nor coccoidal—can be shown to be endowed with fertility it is clearly not an involution form in any reasonable sense of that ill-used word. And yet till



1916 there were no adequate observations on record that this, the supreme test of bacteriological science, had been applied to the lower bacteria to determine whether the current belief in their ability to divide by equal binary fission only was, or was not, well founded: though in the case of other organisms, the existence of higher orders of which appeared thus to be established, the test had already been successfully applied. No effort, in short, was made by applying scientific methods of precision to separate false involution forms from genuine.

For example within the past 25 years several papers have appeared dealing with aberrant morphological types of the lower bacteria, some of the authors, such as Lehmann and Neumann in 1896, on insufficient evidence regarding the occurrence of branching and of budding as a phase of normal development. Others, such as A. Fischer in 1897, and Migula in 1900, regarded the occurrence, again without offering satisfactory evidence, as a pathological process induced by cultivation in unsuitable media. Others again record their observations without comment, or without offering any evidence allowing of interpretation of the results shown. In most of the recorded observations the one constant factor in the media employed for demonstrating morphological aberrancies, indiscriminately classed as involution forms, has apparently been the presence of free H ions, both from organic salts and from inorganic, or the addition of relatively non-toxic doses of the various aniline dyes, perhaps acting in the same direction. Thus Vedder and Duval in 1901 noted that in cultures of dysentery bacilli on glucose agar aberrant types were sometimes found which, without further investigation, they thought were exclusively involutionary in character. In 1900 Fischer recorded that he had placed the *V. cholerae*, the *B. anthracis*, and other organisms in hypotonic solutions of salt in water containing glycerin, and he described a condition to which he gave the term plasmoptysis. Spherical swellings appeared, anywhere in the bacillary axis, filled with plasma extruded from the mother-cell, the plasma pushing the cell-wall, at a weakened spot, he said, in front of it. Some of these spherical swellings gradually expanded and faded from view after varying periods of immersion. In these extreme cases there can be little doubt that the process was the result of a lethal disturbance of osmotic equilibrium set up by prolonged immersion in a fluid with a lower concentration of solutes outside the cell than within it. But Fischer produced no evidence that organisms undergoing lesser degrees of plasmoptysis, in virtue of immersion for shorter periods of time, had lost their reproductive powers. And he produced no evidence to show whether the new spherical bodies produced by plasmoptysis were themselves fertile or sterile. And he therefore provisionally concluded that all degrees of plasmoptysis are necessarily involutionary in character, although he admits that he had not tested the point. To this view also Abbott and Gildersleeve, writing in 1904, in an essay marked by sound critical acumen, were also inclined in their explanation of the occurrence of branching and budding forms in their acid cultures of the *B. diphtheriae*:

though they too were careful to state that they had been unable to determine whether their buds and branches were or were not capable of producing a new race, a criterion to which they evidently attached great importance. In 1904 Ainley Walker and Murray noted the occurrence of branching forms of the *B. typhosus* in media containing gentian violet, fuchsin-methyl green, or methylene blue, and in 1912 Revis dealt with the cultivation of coliform organisms in media containing malachite green. In addition to these there are numerous other recorded observations of morphological aberrations, notably those by Almquist, by Sopp, and by Norsk, to which access has so far not been possible, by Horrocks in 1911, and by Löhnis in 1916.

In all these papers however, with the possible exception of those not yet studied, there is, as stated, no single observation on record that any of these branching or budding forms had been isolated with a view to determining not only their own fertility, but also that of the buds and branches themselves, either before or after their separation from the mother-cell. In addition to all these observations on the occurrence of plasmoptysis phenomena it has not infrequently been noted that under certain conditions plasmolytic changes may be set up in bacteria, leading to endo-fragmentation of chromatin. In bacilli and in cocci so affected, and the change may also be demonstrated in streptococci and streptobacilli, minute dots of deeply-staining material make their appearance. This can often be readily made out in unstained living preparations, which may also contain these bodies in large numbers after extrusion. Photographs of drawings of these bodies were shown by me to the Royal Society in 1916. This endo-fragmentation, described by Fischer in connection with his studies on bacteriolytic serums, has again generally been looked upon as a purely involutionary phenomenon, there being again no recorded warm-stage observations to determine the sterility or fertility of these minute bodies after extrusion from the mother-cell.

#### PHYSIOLOGICAL AND PATHOLOGICAL DEGREES OF PLASMOPTYSIS AND PLASMOLYSIS.

From what has been said it is clearly a matter of fundamental importance to determine whether the occurrence of plasmoptysis in bacteria is a pathological process only, or whether it is, if disturbance of osmotic control be not too profound, a genuine physiological process. Demonstration of unfamiliar methods of reproduction of organisms undergoing moderate degrees of plasmoptysis, combined with demonstration of the fertility of the new race, would unquestionably prove that the phenomenon does represent a phase in physiological development when environmental conditions become difficult, and, incidentally, that bacteria can reproduce themselves in more ways than one. The problem as to the exact point at which physiological processes end, and pathological processes begin, may safely be left to the curious in such matters. It also appears to be a matter of equal importance to determine whether the occurrence in bacteria of the opposite phenomenon of plasmolysis—induced



by osmotic disturbances set up by a lower concentration of solutes within the cell than outside it—is a pathological process only: or whether it too, when kept within proper limits, does not represent a phase in physiological development.

In the case of plasmoptysis where the crucial point in distinguishing between physiological and pathological events appears to be the potential fertility of the extruded plasma in its new cell-wall, so in the case of plasmolysis the crucial point in giving the correct answer appears to lie in proving or disproving the potential fertility of fragmented chromatin within the cell itself, or outside it. In the former case, that of plasmoptysis, the extruded elements would appear to be the expression of exogenous methods of reproduction by unequal binary fission, giving rise to fertile branches, buds and spherical bodies. And in the latter case, that of plasmolysis, the fragments of intracellular chromatin would appear to be the expression of endogenous reproductive activity equivalent in effect to the exhibition of multiple gonidia: the fertility of the new bodies produced both by exogenous and by endogenous methods of reproduction being finally expressed in the ordinary vegetative forms familiar to laboratory students.

In the appended photographs of drawings will be seen some of the numerous types of organisms observed in 4 per cent. glucose agar, or 4 per cent. glucose broth, cultures from a single colony isolated from the faeces of a severe case of typhoid fever which proved fatal on about the fourteenth day of the disease. Indisputable evidence of the direct relationship of many of these forms to the *B. typhosus* is given below.

#### PLATE IV.

The drawings in Plate IV represent composite selected fields, that is to say, they do not represent fields as actually observed, each drawn field containing selected organisms from numerous fields, the basis for selection for each field being merely the type of organism it is desired to illustrate. Each field is designated with a separate label, with the suffix -oid, merely to show how closely many of the types depicted may simulate parasitic fungi, and to facilitate description.

For example:

Field 2 illustrates various types of bacteroid				
„ 3	„	organisms resembling	sporangia	
„ 4	„	„	„	chlamydo-spores
„ 5	„	„	„	oidia
„ 6	„	„	„	gonidia
„ 7	„	„	„	cocci

Fields 1, 2, 3, 4, 5, 9, 10 mainly represent unstained living organisms, drawn, with the exception of 3, by the aid of the camera lucida.

Fields 6, 7, 8 represent stained organisms drawn mostly by freehand.

The following types were found to be highly motile, this motility being repeatedly confirmed during the eight months that this strain was under daily subcultural observation:

Bacteroids	Oidioids
Sporangioids, attached and detached	Gonidioids.
Chlamydo-sporoids	

The only types in which motility was not observed were the thick-walled resting cells depicted in Field 1, and the thin-walled coccoids seen in Field 7. The motility of these there is no record of.

As noted in Plate IV, each field represents organisms observed in cultures on plus 10 agar varying in age between 1 and 4 hours, subcultured from 4 per cent. glucose broth cultures varying in age between 12 and 18 hours. In all cases the maximum incubator temperature employed was 28° C. to 30° C.

The following short description of each field will be sufficient to bring out the points it is desired to emphasize.

*Field 8* is of an ordinary plus 10 broth 8 hour *B. typhosus* culture of a strain which had never been grown in media containing glucose. In addition to "normal" bacilli, and one or two organisms exhibiting Artaud's nodes, are to be seen three deeply-staining forms, two bacillary and one bacteroidal, such as are discussed in full in the text. In these deeply-staining forms, which—as here—may or may not be aberrant in form, lies the key to the problem presented by aberrancy of bacterial form. Hitherto these deeply-staining forms, often known as giant-forms, have been looked upon as involution forms, carrying the suspicion of being sterile forms. Actually they are highly fertile, and may exhibit one or more of many different types of reproductive activity. Possession of this deep receptivity of stain appears in fact to presage an explosion of reproductive activity. These organisms are readily made out in the living unstained condition, and can usually be relied on to demonstrate their fertility on the warm-stage when environmental conditions are suitable.

*Field 2* represents various types of bacteroid, such as found in *B. radicola* and other organisms of agricultural interest, found in cultures of the single strain of *B. typhosus* here studied. The motility of these bacteroid forms was frequently of the rotatory type, the two limbs of the Y at 28° C. revolving with great rapidity. On detachment of a limb this, whilst still short, moves in the ordinary way as does the ordinary bacillus, but as it lengthens it may take on a well-defined serpentine movement. Some of the bacteroids segment terminally, as shown, in oidial fashion, the small detached spheres being themselves highly motile.

*Field 3* represents organisms apparently undergoing the plasmoptysis changes described by Fischer. A normal motile bacillus will sometimes be seen to extrude, anywhere in the bacillary axis, a minute spherical swelling. This gradually expands, the motility of the mother-cell being still unimpaired, progression being in the ordinary way, or by rhythmical serpentine movements. As the sphere expands the mother-cell, with its attached sphere, rapidly rotates, the sphere, if laterally placed, appearing to be presently swung off at a high velocity. Once detached the sphere exhibits a high degree of motility of its own. Its subsequent history is dealt with below. The mother-cell is meanwhile still motile, and may extrude a second, or even a third, new sphere which behaves as before. Sometimes, especially if a sphere has come into contact with another organism, the sphere will remain more or less motionless, whilst the mother-cell will revolve in all planes round the point of attachment to the sphere till finally separation is achieved. If these changes are watched in glucose broth on glucose agar, instead of in normal broth on plus 10 peptone agar, the spheres as formed, presumably on account of the rapidly rising acid tide, will expand and either burst (stained impression films show this well), or will gradually fade from view, either before detachment from the mother-cell, or after detachment, in either case losing their motility.

*Field 4* represents types of organisms which are often figured in text-books as "involution" forms, especially those bearing a superficial resemblance to chlamydospores. There are also figured in this field two ovoids. All the forms here shown are highly motile.

*Field 5* represents terminal and median segmentations of spherical form, occurring in bacilli, and mimicking oidial formation. The separated coccoidal bodies are motile.



*Field 6* shows bacillary, spherical and bacteroid forms undergoing chromatinolysis, the minute fragments of chromatin being frequently observed on the warm-stage in the process of extrusion from the mother-cell. Their subsequent development is dealt with below. Some of the extruded bodies are very minute, often measuring 0.1 to 0.2 microns in their greatest diameter, and able, as such, to pass the coarser filters such as Berkefeld's and Masson's. These minute bodies exhibit high degrees of motility.

*Field 7* shows various sizes of coccoidal bodies, mostly with thin walls, when the stain is taken relatively slightly, contrasting strongly with the thick-walled, deeply-staining coccoids, a few of which are seen in *Field 1*. Photographs of these two types of coccoids are seen in Part II of this paper. It is often said that these coccoid bodies are bacillary bodies seen in cross section. This view is the result of want of their observation on the warm-stage, when they may be readily observed to rotate, if pressure on them be sufficiently light, as definitely spheroidal bodies.

*Field 10* represents, grouped in one field, the various types of organism, which were often seen to be present at the same moment in a true microscopic field at the edge of warm-stage preparations of 4 per cent. glucose broth cultures on plus 10 agar. They were also often seen in subcultures in plus 10 broth from glucose cultures. In this field also are seen four forms resembling spirochaetes. These are large detached flagella.

*Field 11*. These spirochaetoid forms, often tapering to a fine point at each end, with wide spirals, varying in number from 4 to 5 to 70 and 80, the maximum counted (perhaps attached end to end), sometimes stretching across several fields, do not appear to be motile or to segment. They appear in fact to be flagella, attached forms being also shown. They may be seen on the warm-stage to adhere together one by one till thick tresses are formed, such as are sometimes seen in tetanus cultures. They are easily seen unstained, and have been noted in attachment to all the various types of organism shown, including the minute gonidial forms, except to the coccoid forms, and the thick-walled resting cells. The absence of flagella in these forms, appears, as will be seen in Part II, to be associated with non-agglutinability, their development into bacillary forms perhaps going hand in hand with the acquirement of flagella and of agglutinability. It is certain that when development from coccoid to bacillus is complete flagella are present, and that agglutinability is developed, but it has so far proved impossible to watch on the warm-stage the development of flagella *de initio*. In staining preparations of glucose cultures containing large numbers of flagella, attached and detached, it is not necessary to use silver salts. With a preliminary clearing with acetic acid and formalin beautiful preparations are readily made by counter-staining with carbol-fuchsin. In some preparations a fine reticulum may be seen of extremely fine short flagella in dense meshwork formation.

It is necessary to emphasize the fact that all the types of organism shown in Plate IV, except the thick-walled cells, and the large flagella, can frequently be noted in many, but not in all, quite young cultures of the *B. typhosus* in standard media, to which glucose has never been added, provided that patient search be made. It is also necessary to emphasize the fact that the various types of organism shown in Plate IV were again and again found in cultures from single cells, as well as in cultures from single colonies, the precaution always being taken continuously to watch development from single cell to single colony, for purposes of subsequent identification. The necessity for this precaution, and the uselessness in work of this nature of any method of single cell isolation which does not allow of continuous observation from single cell to single colony, will be readily understood by reference to *Field 6*, Plate IV, and *Field 8*, Plate IV. The presence of the minute gonidial bodies there depicted makes it impossible to be certain, in the attempt to isolate a single cell of normal bacillary form and size for example, that a minute gonidium is not at the same time being unconsciously picked up. If therefore the ordinary methods of isolation be employed, such as Barber's method, Malone's method, or the fragmented glass method, methods which

involve inoculation of a liquid medium without opportunity for adequate control observation of growth from single cell to single colony, it may happen, and does happen, that more than one organism is picked up, and that the experiment is ruined. If however a method be adopted which does allow of continuous observation to ensure that a given colony has developed from one cell, and from one cell only, the results obtained can be relied on. No satisfactory method of this nature was available, and one had therefore to be devised. This method is fully described in my paper on pp. 361—368 of this volume of *Journ. of Hygiene*.

## PLATES V AND VI.

These Plates reproduce drawings of continuous warm-stage observations of development from single organisms to single colonies.

In *Series A*, B, C, Plate V, and in *Series I*, Plate VI, is figured the development of bacteroids, unequal binary fission giving rise to ordinary bacilli which, as in Plate V A, may continue to divide by equal binary fission, or, as in Plate V D, again become triradiate, and again undergo unequal binary fission. As a rule only one limb of the Y is thrown off at a time, though segmentation of two or three limbs may go on simultaneously, or in short succession. It sometimes happens that a bacillus will undergo unequal fission without manifesting branching.

In *Series D*, Plate V, is seen the development of bacteroid forms from a diplococcal organism, the exact contour of which was determined by observation of rotatory movements *in situ* before elongation to bacillary form had begun.

In *Series G*, Plate VI, is seen development from a single coccoid form to ordinary bacilli.

In *Series H*, Plate VI, is seen development of a "chlamydosporoid" form, germination, segmentation of the new limb, separation, and germination of a fresh limb, with segmentation by equal binary fission of the limb first formed, being clearly seen. This was a highly motile organism, and no attempt was made to immobilize it in order to obtain a camera lucida drawing for fear of arresting its development, a free supply of oxygen having been found essential to rapid growth and segmentation of similar organisms. A freehand drawing was therefore alone possible. A similar course of events is shown in *Series J*, Plate VI.

In *Series E*, Plate VI, to which the same restriction applies, may be seen the plasmoptysis phenomenon described by Fischer. In this case, however, continuous observation on the warm-stage enabled the observer to determine the fertility and motility of the organism in question, as depicted.

In *Series F*, Plate VI, is shown, at the end of the series, plasmolytic fragmentation of chromatin, followed by extrusion of chromatin, and by development of the highly motile extruded bodies into minute bacilli, each of these after a time exhibiting at each pole one of Artaud's nodes with a clear space between. These rapidly enlarged, though the enlargement is not drawn, under observation, still retaining their motility. These are the minute gonidial bodies, figured in Field 6, Plate IV, as present in, and extruded from, bacillary forms as well as from spheroidal, which can pass coarse bacterial filters, and which can sometimes be seen in standard media, as well as in glucose media, or in media to which HCl has been added, when the requisite alteration of concentration of solutes—in the direction of plasmolysis production—has been reached. And whenever very minute motile bacilli, especially when showing Artaud's nodes, appear in pure cultures of normal sized *B. typhosus* cultures it may safely be concluded that they have arisen in this way from minute extruded gonidial bodies. In order satisfactorily to study the development of these organisms a free supply of oxygen and of moisture is absolutely essential, no development of this nature taking place except at the moist edge of the preparation nearest to a free supply of air.



Having thus shown development from single cell to single colony it was then necessary to identify fully the colonies obtained. Eighteen different aberrant types were isolated, including giant bacillary forms, similar to those seen in Plate IV, Field 8, coccoid forms similar to those seen in Field 7, bacteroids similar to some of those seen in Field 2, chlamydo-sporoids similar to some of those seen in Field 4, sporangiods similar to those seen in Field 3, and resting cells similar to those seen in Field 1.

The broth cultures from each of these were fully identified by cultural, biochemical and serological tests, an agglutination of from 1/20,000 to 1/40,000 being in all cases eventually obtained.

In two cases identification was further completed by absorption tests, and in the case of the cultures handed to the War Office Committee of enquiry absorption and inoculation tests were also successfully passed.

During the course of the work undertaken in connection with study of methods of bacterial reproduction the following facts came chiefly into prominence. Some of these have already been noted by bacterial morphologists.

1. In some cultures of the lower bacteria, whether young or relatively old, whether standard in initial reaction, or containing glucose from which, in the case of the *B. typhosus*, acid is rapidly formed, or deliberately made acid by the addition of free HCl, aberrant forms of organisms have never been found, even after prolonged search. The entire population in such cases appears to consist of "normal" organisms, staining relatively lightly, and dividing by equal binary fission only. The general viability of such a population after a few days in 4 per cent. glucose cultures, or in HCl cultures with an initial reaction to phenolphthalein of plus 20, appears to be considerably lowered, as measured by subculture.

2. Aberrant types of organism are sometimes to be seen in small numbers in quite young cultures in "standard" media. These aberrant types, found in young cultures, sometimes in relatively large numbers, usually stain deeply and uniformly, unless undergoing chromatinolysis, when the fragmented chromatin appears as deeply staining dots, contrasting strongly with the less deeply-staining mother-cell. These aberrant types may be only aberrant in their deep receptivity of stain, there being no departure from the normal bacillus in outline. Other aberrant types, also characterized by uniformly deep receptivity of stain, may be branching or budding forms, or may be coccoid in form, or may simulate parasitic fungi by the formation of bodies resembling sporangia, chlamydo-spores and so forth. All these deeply-staining forms may be highly fertile.

3. These aberrant types, if already present in a standard culture, will increase in number with the age of the culture, or by the addition of glucose, or of free acid, the normal population under such circumstances beginning to disappear until it is again temporarily reinforced by direct descendants from these fertile aberrant forms. The viability of such a culture as a whole is greatly impaired by prolonged immersion in 4 per cent. glucose media, or in media containing an excess of free HCl. The viability of the deeply-staining

aberrant forms referred to is however higher than that of the general population, and it is clear that they have higher resisting powers to acid influence. This is also shown by the fact of their presence in greater number in old cultures than in young, and in the fact that on transference to fresh media the new generation appears to start from them and not from the survivors of the normal population. This can readily be demonstrated by watching the development of individual organisms on the warm-stage. It is quite unnecessary in order to establish the higher resisting powers to acid influence of these selected individuals to show also that they exhibit greater resistance to heat or to antiseptics than do "normal" individuals. This statement is necessitated by attempts that have unsuccessfully been made in the past to establish a higher degree of resistance to heat and to antiseptics in the case of so-called "involution" forms, as compared with normal forms, without any reference to the cardinal fact of their demonstrable fertility.

4. In addition to the fertile deeply-staining forms described there are also often to be seen in young cultures in standard media, as well as in glucose media, lightly-staining aberrant forms. These organisms, distinguishable from involution forms in their symmetry of outline, and in the fact that they do not fade from view on the warm-stage, may be themselves highly fertile. Examples of these in coccoid form are seen in Chart 4, Part II, of this paper.

5. On transference to standard media from glucose cultures, or from HCl cultures, aberrant forms tend rapidly to disappear, the need for their services being perhaps no longer required in early stages of growth on standard media. When, however, the age of these media increases, or when glucose is added, they again tend to reappear. And if careful search be made it will often be found, whilst the cultures are still young, or before glucose has been added, that deeply-staining bacillary forms are here and there present. So long as conditions remain more or less favourable these may divide by equal binary fission only. But when conditions become less favourable they may often be seen to take on unfamiliar methods of reproduction, though this, as shown in Plates V and VI, is also to be witnessed within an hour or two of transference to standard media.

6. Transference of genuine involution forms from old or otherwise unsuitable media to fresh standard media does not restore their uniform receptivity of stain, their symmetry of outline, nor their fertility. In some instances there may be seen to appear in genuine involution forms highly-refractile spherical bodies, suggesting droplets of lipid material, which rapidly increase in size under observation. These bodies do not take the ordinary stains, but stand out as bright clear spaces in the irregularly stained matrix of the cell.

7. If standard broth media with an initial reaction of plus 10 to phenolphthalein be inoculated with acid-producing organisms, such as the *B. typhosus*, and be repeatedly titrated, evidence of a rapidly rising acid tide is forthcoming within two or three hours of incubation. A progressively unfavourable environment is thus rapidly produced which appears to be similar to, and to approxi-



mate to, the unfavourable environment of old cultures, or of cultures to which glucose or free acid has been added. The rapidly increasing concentration in a constant volume, with no provision for their removal, of degradation products from the substrate presented by the constituents of media, and by dead organisms, with a rapidly increasing concentration of organic catalysts no doubt accounts for this rising acid tide in standard media. In attempting therefore to estimate the reciprocal influence of bacteria and their surroundings in terms of morphological results it is obvious that the term "standard medium"—once inoculation has taken place, and once the population has begun greatly to increase—has no actuality.

#### CONCLUSIONS.

In the light of the facts here recorded it would appear that from the point of view of perpetuation of the lower bacteria through long periods of time, in laboratory cultures at least, the organisms usually regarded as "normal," normal, that is, in form, in their relatively slight capacity to retain the stain, and in their exclusive ability—exclusive so long only as conditions remain favourable—to divide by equal binary fission, represent the least important members of a total population. These "normal" organisms occur in the largest numbers in a given culture when the reaction to phenolphthalein approximates to the neutral point: and it appears that in some cultures they represent the entire population. When, however, as the result of rapid increase in numbers, the circumstances of life become more and more adverse the onus of carrying on the race appears to be chiefly laid on those deeply-staining highly fertile organisms which, from too ready an assumption of their sterility, have hitherto been indiscriminately ranged with genuine involution forms. From the point of view of perpetuation of the race as a whole these deeply-staining organisms, sometimes aberrant in form and sometimes not, able to produce fresh bacilli now by this method of reproduction, now by that, according to the osmotic needs of the moment, are incomparably the most important. In the history of bacteria of the lower orders it would appear that the existence of these deeply-staining organisms, and of the various types of reproductive activity which they exhibit, is a direct expression of the reciprocal influence through the ages of bacteria and their surroundings in terms of selective adaptation to the vicissitudes of bacterial life (unless indeed it be alternatively suggested that they represent a non-bacterial order, with equally dividing "bacteria" as a vegetative side-issue, as from their mimicry of the parasitic fungi one was tempted to surmise). And if it were not for these deeply-staining forms, with relatively high viability in acid media, it would be difficult to see how cultures of non-sporing bacteria could survive in the test-tube for long periods of time if reliance could only be placed on "normal" individuals with the lower degree of viability which is unquestionably imposed on them by prolonged residence in progressively adverse surroundings. It must not however be supposed that the process of selective adaptability is one which

can be followed at will in the laboratory and that "forms identical with the aberrant forms here depicted have arisen in definite response to the conditions experimentally provided, and may be expected constantly to reappear whenever the same environmental conditions are reproduced," as suggested by the Committee selected to study the facts presented. Because this is precisely what does not happen. As already stated these deeply-staining aberrant fertile organisms only appear to increase in cultures already containing them, as for example in cultures in which circumstance has deliberately been made adverse. They emphatically do not appear, within the limits of observation, in populations exclusively composed of normal organisms when the same adverse circumstances are introduced. One can only conclude therefore that in these selected individuals there resides, as the result of far distant training, the inherited faculty of reproduction by unfamiliar methods when necessity arises. And these methods are only unfamiliar because this has not been recognized, and because they have not been looked for.

#### NOTE.

This Committee consisted of Colonel Sir William B. Leishman, Colonel Professor J. G. Adami, Professor J. B. Farmer and Lieut.-Colonel D. Harvey.

The subject of enquiry was presented by me in a typed memorandum in the following terms:

"The sole objective of the present enquiry is determination of the complexity or otherwise of the life-cycle of the causal organism of Typhoid Fever, in so far as this is amenable to study in unfiltered laboratory cultures. We are therefore here concerned with one question only—Is the *B. typhosus*, or is it not, capable of being produced in any other way whatever beyond that of equal transverse binary fission of a pre-existing *B. typhosus*?"

During their work this Committee applied to my cultures the ordinary routine tests for purity, including absorption and inoculation tests. And they reported that they fully endorsed my statement that the cultures were pure cultures. They did not however apply the crucial test of purity which I had myself applied, namely that of isolation of single aberrant cells, and of continuous observation on the warm-stage of growth from single cell to single colony, with subsequent identification of the new races thus produced. They also fully confirmed by personal observation of warm-stage development my claim that the *B. typhosus* can be reproduced from non-bacillary forms by unequal binary fission, as well as from bacillary forms by the ordinary method of equal binary fission. Confirmation by independent observers of my main thesis, as specifically presented, was thus complete. It may therefore be unequivocally stated that it has now been definitely proved, and accepted, that the lower bacteria, as illustrated by the *B. typhosus*, can and do reproduce themselves in more ways than one, and that conse-



quently the reproductive life of this organism is not necessarily so simple as it has hitherto been assumed to be.

The Committee, I think wisely, deprecate the use of the term life-cycle in connection with the reproductive life of the individual bacterium as an organism which inevitably, sooner or later, as a necessary stage in the completion of its life-story must—in the case, for example, of a bacillus—pass through a non-bacillary phase. In the case of bacteria placed under the artificially favourable environment supplied for short periods of time by culture media with an initial standard reaction the life-story, such as it is, is unquestionably simple and not complex. But it is not sufficient to study the morphology of bacteria in the short periods when circumstance is easy. It must never be forgotten that this initial state of favourable circumstance is always fugitive in nature, in infected tissues, and in the test-tube, once the population has begun rapidly to increase in a confined space, with no provision for removal of degradation products. It is this progressively unfavourable environment which appears to have evoked a selective adaptation in virtue of which unfamiliar methods of reproduction come into play to ensure permanence of the race through long periods of time. To say therefore that in the lower bacteria the reproductive life is sometimes not simple but complex is indisputably true, because to preserve the race in terms of time a bacillus, for example, may have eventually to pass through a non-bacillary phase.

The confirmation, above referred to, by this Committee of the complexity of bacterial reproductive life under certain circumstances was however at once qualified by an evident reluctance to abandon the idea that the results demonstrated were the expression of an involutionary process, and had in consequence little or no bearing on the practice of bacteriological science.

In support of their view that involutionary processes could still be invoked to explain the results the Committee referred to the work of Fischer, and of Abbott and Gildersleeve, under the evident impression that the plasmoptysis of Fischer was looked upon by these authors as necessarily a pathological process. Fischer however, as well as Abbott and Gildersleeve, specifically state that they had had no opportunity of determining the fertility or sterility of the new bodies produced by plasmoptysis, a distinction which these authors clearly recognized to be one of fundamental importance. And their lack of opportunity of settling this point appears to have been due to the fact that they knew of no reliable method of isolating organisms for the purpose, a deficiency which I had been at great pains to make good. In the absence therefore of demonstration of the fertility of these organisms these authors had no alternative but provisionally to assume that they were sterile, and to conjecture that all degrees of plasmoptysis are therefore involutionary in nature. The bacteriologists of the Committee however in their official report make no reference to this qualification of Fischer's work by Fischer himself, and by Abbott and Gildersleeve, although they had my own positive evidence of fertility before them, which indeed they confirmed by their own observations.

In their reference therefore to this earlier work as confirmatory of their own view they must, doubtless under the stress of war work, either have failed to note that the crucial point of fertility had not been tested in this earlier work—this being the vital distinction between my work and that of previous observers, a distinction which they also omitted to record—or they had not recognized that demonstration of the fertility of new races abnormally produced is fatal to the involution theory, as generally understood. In either case, as they are careful to avoid the use of the word involution, it is necessary to point out that organisms undergoing pathological degrees of plasmolysis and plasmoptysis are only involution forms under another name, and that there appear to be physiological degrees of these phenomena resulting from disturbances of normal restraint of osmotic changes as well as pathological degrees of the same. It is indeed difficult to see how the occurrence of bacterial branching and budding, and of endogenous gonidia production, can be anything else but an expression of restrained plasmoptysis and plasmolysis, assuming, that is, that disturbance of the power of adjustment of osmotic variations within the cell and outside it is the true explanation of the various types of morphological aberrancy here illustrated—an assumption on which few, perhaps, would care at present to dogmatize.

## PART II.

### THE EFFECT OF THE COMPLEXITY OF THE REPRODUCTIVE LIFE OF BACTERIA ON THE AGGLUTINABILITY OF BACTERIAL EMULSIONS.

Belief in the ability of the non-sporing bacteria to divide under all circumstances by equal binary fission only is reflected in bacteriological practice, especially as regards determination of the precise etiology of infections, the methods employed for identification of bacteria, and the theory of the multiplicity of bacterial strains. It also enters largely into prophylaxis and treatment with specific serums and vaccines, and is an essential feature in the detection and control of bacterial carriers, as well as in epidemiological work, including statistical analyses. Now however that it is known that the life-story of bacteria, even in standard laboratory media, is one of great complexity, and that their reproductive life faithfully reflects the progressive changes of milieu inseparable from laboratory cultivation it becomes necessary to examine the various bacteriological problems referred to from a different point of view.

In the present communication I propose only to deal with the problem presented by serological reactions as an aid to identification of bacteria, and in doing so to show that it is hopeless to expect to obtain constant agglutination results unless due regard be paid to the morphological status of a bacterial population, both when used as an antigen for the production of agglutinin, and when tested for its power of provoking a specific reaction.



I propose, in other words, to show that necessary as attempts at standardization are in terms of initial reaction, or the opacity of an emulsion, or in terms of constancy of culture medium composition, one cardinal feature at least in standardizing serological reactions is determination of a standard morphological equivalent. Unless this be fully taken into account agglutination units have but little practical significance.

It has of course long been known that bacillary organisms of the lower orders of bacteria may occasionally take on a coccoidal phase, and that this may be associated with a deficiency in agglutinability by the specific serum. And there are well known to occur various vagaries of agglutinability, hitherto unexplained, such for example, as the loss of agglutinability on subculture to an agar slope, with subsequent recovery on again restoring the strain to broth. But, as a rule, the current conception of the simple life-history of bacteria has dominated the identification work of bacteriologists, and has prevented any systematic examination of the morphology of bacterial emulsions which might be expected to agglutinate with a given serum, but which nevertheless do not do so. And in consequence there has arisen, especially in bacillary dysentery, a heterogeneous collection of new "strains," marked by strange symbols, justification for the existence of at least some of which rests on the slenderest evidence. It is not possible as yet, owing to the bewildering complexity of the study of bacterial morphology, absolutely to prove that some of these many strains only represent different morphological phases of one strain, difference in form being associated with difference in antigenic values. Nor is it desired to insist that standardization in terms of morphology is the only cardinal factor to be considered in the standardization of bacteriological methods of serological aids to identification. But, as will be seen, there is abundant evidence to show that the morphological factor is one which demands the most searching study, which no bacteriologist can afford to set aside as unnecessary if the standardization of bacteriological methods generally is to be put on a scientific footing.

The value of the agglutination results here recorded entirely rests on the reliability of the technique employed. It is therefore necessary to give a series of control observations to show that constant results were uniformly obtained in using this technique when an emulsion of the same morphological equivalent was put up against its specific serum.

#### CONTROL CHART.

The technique employed in all cases was as follows. The method of reading was macroscopic, often confirmed by microscopic, or by hand-lens, examinations. Distribution of equal volumes of saline, serum and emulsion was made with a new graduated Pasteur pipette for each set of tubes used, the pipette being thoroughly washed and drained between each distribution of material. Dilutions in all cases began at 1/80, except when otherwise stated, the dilution in each case except the first being double that in the preceding tube. Twelve

hour cultures were uniformly employed, the test emulsions being put up without killing the organisms. In the case of *B. typhosus* cultures the plugged tubes were placed in a hot chamber for one hour at 56° C., after which time they were allowed to stand for one hour at room temperature when a first reading was taken, a second reading being taken 12 or 24 hours later. In the case of dysentery cultures the tubes were kept for 24 hours at 56° C. No attempt was made to standardize in terms of opacity, a deliberate omission in order to determine the effect of the omission on the control observations. As will be seen in Chart A the effect was nil. A control tube containing saline and emulsion only was put up for each set of observations in all the experiments.

The first set of observations deals with typhoid cultures. This set is divided into the following groups.

#### CHART 1, GROUP A.

In the case of strain Pierce, morphological variations in which formed the subject of Part I of this paper, it was found that the thick-walled coccoids there referred to, *vide* Plate VII, figure 1, were non-agglutinable. It was also found that under the influence of the acid produced from the glucose added to the cultures these thick-walled coccoids represented a resistant resting phase, and occurred in a bacillary population which was itself fully agglutinable. It was also found by isolation of one of these thick-walled cells that it germinated, and gave rise to large bacillary forms, which in their turn gave rise to forms which were again fully agglutinable. The process from agglutinable bacillus to non-agglutinable thick-walled cell and back again to agglutinable cell was therefore reversible. The results obtained are expressed in graphic form in Chart 1, which also gives the key to cultures submitted to agglutination tests, together with the dominant morphological equivalent for each culture. Photographs of these thick-walled coccoids are appended. It is noticeable that emulsions 4 and 8 were from glucose agar slopes.

#### CHART 1, GROUP B.

From the same strain Pierce subcultures from glucose broth were made as shown in the chart. In cultures 1 and 2 the dominant population was composed of thin-walled lightly-staining coccoids. Photographs of the actual emulsions of these coccoids used in these agglutination tests are given, together with a photograph of the mixed coccoid and bacillary emulsion 3, which agglutinated to 1/1280. The remaining emulsions 4, 5, 6 were mainly bacillary, as also was the original emulsion A, all of these agglutinating to high titre. It is again to be observed that the non-agglutinable emulsions 1 and 2 were from an agar slope, and from a glucose agar slope. Since recovery of agglutinability ensued on subculture in broth, *vide* cultures 4 and 5, associated with reversal to bacillary form, it might be thought that the appearance of a coccoidal non-agglutinable phase is the result of cultivation on a solid medium. That this is not necessarily the case is shown in Group C, where the non-agglutinable phase is seen to persist in broth culture.

#### CHART 1, GROUP C.

From the key to subcultures 1, 2, 3, 4, 5 from the same Pierce strain it will be seen, Chart 1, Group C, that the non-agglutinable coccoidal phase persists in broth cultures 2 and 5 with temporary reversion to agglutinable bacillary phase in broth culture 3, followed by return to coccoidal non-agglutinable phase on agar, culture 4: the same persistence of this in broth, culture 5, from the original agar, being again seen.



## CHART 1, GROUP D.

Having thus found in Groups A, B, C that, using the stock serum issued by the Lister Institute, a dominant coccoid population in a selected *B. typhosus* strain subcultured from glucose cultures, will show little or no signs of agglutination, whilst bacillary subcultures from single isolated coccoids (*vide* Plate V, Part I) may agglutinate in a dilution of 1/40,960, the same strain Pierce was then tested in parallel with an R.A.M.C. stock serum, and with the same Lister stock serum as before; two colonies being subcultured from the same agar plate, for purposes of comparison, into glucose broth, each tube of this being further subcultured as shown in the key. It was not found possible, at the date on which this experiment was carried out, to provide at the required moment a subculture from strain Pierce with as dominant a population of thin-walled coccoids as shown in Groups C and D. But a sufficient number were demonstrable to produce wide variations in the agglutination results obtained between the readings respectively of dominant coccoidal and dominant bacillary populations, with intermediate readings, discussion of which is for the moment deferred. In addition to this the following points of interest emerge from study of Group D.

A. Subculture 7, in which the coccoidal population was dominant, agglutinated only to 1/320 with both the Lister and the R.A.M.C. serums, in the case of colony A, and only to 1/160 with both serums, in the case of colony B. The amount in both the Lister and the R.A.M.C. serums of specific agglutinin to the coccoid phase of the *B. typhosus* would appear therefore to be very small. In the case of the Lister serum this had already strongly been suggested in groups A, B and C. The great similarity in the agglutination results in the case of subcultures 7, as regards both colonies A and B, also indicate that the course of development in the case of both these colonies, as shown in the particular subculture 7, was approximately the same. And this was confirmed by the dominant coccoid population in both. So far the distinction between coccoid non-agglutinable and bacillary agglutinable phases, already illustrated in groups A, B and C, appears to be a simple matter, when considered in the light of results obtained by using subcultures from a single colony tested against one serum.

B. When however further controls are established, as by testing subcultures from two colonies against two serums in parallel, most discordant results emerge, which suggest that it is not merely a question of the relative percentages of coccoidal and bacillary members of a population. For example, subculture 10, colony A, gives a full titre of 1/20,480 with both Lister and R.A.M.C. serums, whilst subculture 10, colony B, gives a full titre of 1/20,480 with Lister serum, but a titre only of 1/1280 with R.A.M.C. serum. Again, subcultures 1, 2, 4, 6, 8, 9, colony A, give full titre with Lister serum, but much smaller titres with R.A.M.C. serum: whilst, allowing for the smaller number of subcultures tested in the case of colony B, there is again great similarity in the results obtained with Lister serum, but great disparity in the results obtained with R.A.M.C. serum. Imperfect observation of the agglutination results affords one way of escape from the dilemma. This is however excluded by the control results given at the opening of Part II of this paper. Contamination of the cultures affords a second way of escape. This however is practically excluded by the fact that cultures 1, 2, 4, 6, 8, 9, 10 agglutinated to 1/20,480 with Lister serum in the case of colony A, and that cultures 1, 2, 8, 9, 10 agglutinated to the same titre with the same serum in the case of colony B. Still a third way of escape is offered by the theory, upheld by many, that any given bacterial culture may be composed of numerous strains, and that some subcultures from such cultures may exalt this strain, and other subcultures that strain. This way of escape also appears to be closed by the fact, demonstrated in Part I, that organisms appearing to represent different strains in one culture can develop within a few hours from a single cell. The true explanation in fact appears to lie in this demonstration, though it is not suggested that other factors, as yet

undetermined, may not be partly responsible for the agglutination vagaries here illustrated. Confirmation of the view that each agglutination departure from full titre corresponds with a definite morphological equivalent was obtained by careful examination of subcultures 1, 2, 4, 6, 8, 9, 10 when it was found that:

(1) Repeated replating gave no indication of contamination.

(2) Culturally, biochemically and serologically (in terms of Lister serum) they were genuine *B. typhosus* cultures.

(3) Morphologically some of the cultures contained in addition to "normal" bacilli large numbers of forms of bewildering variety, the relative percentage numbers of these—which were mostly bacillary—varying with each subculture. In addition, for example, to coccoids and to "normal" organisms would be seen bacilli considerably larger, and very much smaller, than the normal, some exclusively bacillary, some cocco-bacillary, others undergoing buckling or branching, others again undergoing chromatinolysis. Some had pointed ends, others had rounded ends: some were small, exhibiting Artaud's nodes, others were considerably larger, also exhibiting Artaud's nodes. Some were thick-walled bacilli, taking the stain deeply, others, again, of the various types described took the stain relatively lightly.

The amount of work involved in attempting to obtain an accurate idea of the relative proportion of each of these various types in a large number of cultures exhibiting intermediate degrees of agglutinability would clearly be prohibitive, without a trained staff of workers. No attempt therefore was made in the observations now to be recorded to interpret in morphological terms varying degrees of agglutinability of different subcultures of the same culture when tested either with one serum, or with two or more serums in parallel: the main object being merely to show that standardization in terms of morphology must in the future be comprehensively studied if constantly reliable results are to be obtained.

#### CHART 1, GROUP E.

That some serums appear to have been unconsciously prepared with polyvalent antigens, polyvalent that is in the sense of polyvalency of morphological types and not in the sense of polyvalency of "strains," is shown by Group E. In this group subcultures from the same strain Pierce were tested against the Oxford stock serum, and against the R.A.M.C. serum. The results, as before, with the R.A.M.C. serum are poor, whilst the results with the Oxford serum, against the Pierce strain, are very good, suggesting lack of morphological polyvalency in the former serum, and its presence in the case of the latter serum.

So far tests have been confined to subcultures of the strain of *B. typhosus* from glucose cultures, which appear to be particularly favourable to the initiation of different types of morphological development. To this extent therefore the tests so far applied would appear to be unnecessarily severe, and perhaps of little practical value unless confirmed by similar results obtained from tests applied to subcultures from ordinary cultures to which glucose has not been added. Tests were therefore applied to subcultures from ordinary cultures, in order to determine if similar vagaries of agglutination occurred, with the following results.

#### CHART 2, GROUP F.

Here considerably better results are obtained, though even with such good serums as the Oxford and Lister serums marked variations in the titres given with each of these occur. Taking the results given with the three serums the minimum occurs with the R.A.M.C. serum of 1/640, and the maximum with the Lister serum of 1/40,960.



## DYSENTERY CULTURES.

## CHART 3, GROUP G.

As shown in this chart four subcultures from 12 hour agar cultures of Lister stock strains F and Y were tested respectively against Lister F and Y stock serums, cross tests being also carried out. In this experiment the condensation water in each agar tube was replaced by broth, cultures 2 and 4 representing the broth substitute for condensation water in cultures 1 and 3. The object of this arrangement was merely to show that different morphological results, with different morphological readings, can be shown in one test-tube according to whether the agar slope, or the broth at the bottom of the tube, be inoculated at the same moment from the same source: care of course being taken to keep the tubes vertical during incubation, and to ensure that in removing the broth culture no admixture of this with the culture on the surface of the agar slope not covered by broth takes place. This agar culture, after removal of the broth, was itself removed with a sterile glass rod from the upper half only of the slope, and was then emulsified in sterile broth: both cultures being then at once tested. Study of the chart shows that:

1. The *B. Flexner* broth subculture 4 does not agglutinate at all, in any of the given dilutions, either with Flexner serum, or with Y serum: whilst the *B. Y* broth culture 4 agglutinates to 1/160 with Flexner serum, and to 1/320 with Y serum. The approximate respective morphological picture in each of these cultures is given in the chart.

2. The *B. Flexner* agar subculture 3 agglutinates to 1/640, with Flexner serum, and to 1/160 with Y serum: whilst the *B. Y* agar subculture 3 agglutinates to 1/320 with Flexner serum, and to 1/320 with Y serum.

3. *B. Flexner* agar subculture 1 agglutinates to 1/160 with both Flexner serum and Y serum, whilst *B. Y* agar subculture 1 agglutinates to 1/640 with Flexner serum, and not at all with Y serum.

4. *B. Flexner* broth subculture 2 does not agglutinate at all, in any of the dilutions, with Flexner serum, but to 1/1280 with Y serum: whilst *B. Y* broth subculture 2 does not agglutinate at all, in any of the given dilutions, with Flexner serum, and to 1/640 with Y serum.

Thus, to take an extreme example, agglutination of *B. Y* agar subculture 1 would suggest that this was a culture of *B. Flexner*, other tests being in the same direction, the figures for agglutination being 1/640 with Flexner serum, and nil with Y serum: whilst agglutination of *B. Y* broth subculture 2 would suggest that this was a culture of *B. Y*, the figures being nil with Flexner serum, and 1/1640 with Y serum. Taking the agglutination results of all four subcultures as a whole an empirical agglutination mean, E.A.M., can be obtained for each set of four observations with one serum by dividing the total length of agglutination lines by the number of subcultures examined.

The results may then be expressed:

*B. Flexner* versus Flexner serum = 10 E.A.M.

	"	"	Y	"	= 13	"
<i>B. Y</i>	"	"	Flexner	"	= 16	"
	"	"	Y	"	= 13	"

The agglutination results, in other words, suggest that, taking the subcultures as a whole, *B. Flexner* agglutinates better with Y serum than with its own serum, and that *B. Y* agglutinates better with Flexner serum than with Y serum.

Unfortunately reliable sets of sugars were not available when these experiments were carried out: so no opportunity occurred of determining if the results obtained were associated with any biochemical variations. Observations since undertaken, indicating in some cases association of biochemical variation with serological, will be published separately.

## Reproduction of Aerobic Bacteria

### CHART 3, GROUP H.

Observations were then carried out with R.A.M.C. subcultures from stock Flexner and Y cultures, kindly supplied by Lt.-Colonel Harvey, tested against R.A.M.C. stock Flexner and Y serums.

The massed results obtained with six subcultures, instead of four, show a better specificity of reaction in the case of R.A.M.C. cultures and serums than in the case of Lister cultures and serums, the figures being:

<i>B. Flexner</i> versus Flexner serum = 15 E.A.M.					
"	"	Y	"	= 10	"
B. Y	"	Flexner	"	= 6	"
"	"	Y	"	= 17	"

A considerable variation is, however, to be noted in each group between the minimum and the maximum dilutions at which agglutination occurred.

### CHART 3, GROUP I.

The R.A.M.C. stock *B. Flexner* culture used for the observations recorded in Group G, was kept in a sealed tube for over three months at 5° C., and was again subcultured as shown in Group H, several replating observations being made to ensure the absence of contamination. The object of the experiment was to determine the effect of subcultivation from an old culture in the direction of inducing in these subcultures a greater, or a lesser, variation in their agglutination figures than in the case of subcultures from young cultures, as in Group G. At the same time it was desired to test these subcultures against a Lister Flexner serum, and against a Lister F Y serum.

For purposes of comparison the figures from A in Group G are reproduced in the present Group H. The E.A.M. for the R.A.M.C. Flexner subcultures tested with R.A.M.C. Flexner serum is now seen to rise from 15 to 42, the maximum titre supplied by the makers being greatly exceeded in the case of cultures 1, 3, 2, 6. The E.A.M. for the same subcultures tested with Lister Flexner serum C 94 is only 5, three of the subcultures giving no reaction at all. The E.A.M. for these subcultures tested with Lister Flexner Y serum, is the much better figure 32, which is of particular interest in view of the fact that this serum appears to have been prepared by inoculation of F and Y antigens, Lister F and Y cultures having been shown in Group A to be more or less interchangeable.

The maximum variations, as for example B—between 1/160 and 1/20,480, again show how nearly subculture 4, for example, came to the minimum diagnostic titre, even when tested against so good a Flexner serum as R.A.M.C. serum F 7.

### CHART 3, GROUPS K<sub>1</sub>, K<sub>2</sub>, K<sub>3</sub>.

This chart illustrates the discordant results obtained when the R.A.M.C. stock Flexner culture was replated, half of a colony being subcultured on to agar, the other half being subcultured into broth, at the same moment. The same Flexner culture was replated eight times, each time on a series of five plates. Each series was lettered in succession, A, B, C, D, E, F, G, H, a single colony from plates 4 or 5 from each series C, D, E, F, G, H being divided as described. There was an interval of three days between each replating, the original culture remaining at room temperature in the interval.

### CHART 4, GROUP L<sub>1</sub>.

This chart illustrates the results obtained by testing subcultures, for the most part at random—and not entirely, as hitherto, in groups, from a Y culture, obtained from the Kitchener Hospital, Brighton, against different Y and F Y serums, and against a polyvalent serum. As will be seen the agglutination figures obtained do not, taken as a whole,



in this case indicate a marked advantage in favour of the R.A.M.C. polyvalent serum employed, wide variations, from 1/160 to 1/10,240, of readings occurring, in the case of five subcultures from the original agar when tested with this serum.

#### CHART 4, GROUP L<sub>2</sub>.

In this experiment a normal polyvalent serum, R.A.M.C. polyvalent Flexner Y serum D<sub>1</sub> was tested against:

- a. The same Y strain as used in Chart 4, L<sub>1</sub>.
- b. A second Y strain, also from the Kitchener Hospital.
- c. The stock R.A.M.C. Flexner strain, Ledingham, employed in the earlier experiments.

It was desired to determine what variation, if any, occurred when a different medium was employed, such as the tryptic agar medium recommended for meningococcus work.

As will be seen the results obtained with this polyvalent F Y serum were very good, and more constant. The results obtained with agar and with tryptic agar subcultures are also very similar, indicating that the change of medium has little or no effect in producing different degrees of agglutinability.

#### CHART 5, GROUP M.

Attention was now turned to a stock Shiga-Kruse culture, kindly supplied for the purpose by Professor Dreyer's staff at Oxford, subcultures from this being tested against a stock R.A.M.C. Shiga serum, and a stock Lister Shiga serum.

The results obtained may be summarized as follows:

1. *With Oxford Shiga serum.*

- (a) Subcultures 13, 14, 15 give a uniform figure of 1/40,960, suggesting that this is an ideal serum.
- (b) Subcultures 19, 20, 21 from the original agar slope—which had been standing for four days at room temperature—now uniformly declined to agglutinate in a higher dilution than 1/80.
- (c) Subculture 25 from subculture 19 still persisted in refusing to agglutinate, whilst subculture 28 from subculture 20 agglutinated in a dilution of 1/2,560, subculture 27 from subculture 21 now agglutinating in a dilution of 1/40,960.
- (d) Subcultures 31 and 32 from the original agar slope—which had been standing for six days at room temperature—now both agglutinated in dilutions of 1/20,480.

2. *With Lister serum.*

- (a) Subcultures 13, 14, 15 which all gave a figure of 1/40,960 with Oxford serum gave with Lister serum figures of 1/80, 1/160 and 1/320, suggesting that the phases agglutinable by Oxford serum are not represented by specific agglutinins in Lister serum.
- (b) Subcultures 25, 27, 28 now agglutinate to 1/10,240 with Lister serum, suggesting that agglutinins specific to the phases agglutinated by Lister serum are absent in Oxford serum, in so far as subculture 25 is concerned, and are partly absent in so far as subculture 28 is concerned.

3. *With R.A.M.C. serum.*

Similar results as with Oxford serum, and with Lister serum.

From the table showing the maximum variations obtained with each serum it appears that of eleven subcultures tested respectively with R.A.M.C. serum, and with Oxford

serum, three subcultures in the first case, and four in the second case, failed to give any diagnostic readings whilst with the Lister serum two subcultures out of six failed to give reliable diagnostic readings.

#### CHART 6, GROUP N.

Attention was finally turned to *Meningococcus*, Type 1, subcultures from this "strain" being tested in parallel, *vide* Chart 6, against the four Type serums. If the morphology table is referred to, together with the serological table, it will be seen that the results obtained are suggestive. The acquisition by one "strain" of agglutinability by meningococcal serums specific to other "strains" has more than once in recent months been noted, though no satisfactory explanation has been forthcoming. The results shown in Chart 6 suggest however that the explanation lies in the existence of a morphological equivalent for each so-called strain of meningococci, and that the four meningococcal "strains" (meningococcal and parameningococcal) represent developmental phases of one strain only. That the developmental changes to be seen in meningococcal cultures are as complex as those demonstrated by warm-stage observations in Part I of this paper in the case of organisms of the enteric group I fully demonstrated in 1917, also by warm-stage observations (*vide Brit. Med. Journ.* Sept. 22, 1917). That one was perhaps then misled into regarding the unquestionable occurrence of budding, and of endofragmentation of chromatin, in the so-called giant-cells as indications that these organisms belong to the parasitic fungi does not alter the basic fact of demonstration of the fertility of the buds and of the fragments of chromatin thus produced. Nor does this affect the further fact that meningococci were shown on the warm-stage to be produced both vegetatively from pre-existing meningococci, and by chromatinolysis from giant-cells which had till then been believed to represent sterile involution forms. And the fact that in the case of the enteric group of organisms some morphological phases of development have in Part I of this paper been indisputably proved to be associated with agglutination vagaries lends collateral support to the direct evidence produced in Chart 6 that what is true of the enteric organisms is also true of the meningococcus. It appears to be a reasonable view that in the case of the enteric organisms the occurrence of budding and of branching, and of the production of gonidia, is an expression of physiological degrees respectively of plasmoptysis and plasmolysis, operating even in young standard media. And this appears also to be a reasonable explanation of the occurrence of budding in "giant" cells in meningococcal cultures under environmental conditions favourable to the induction of moderate degrees of plasmoptysis, and of the occurrence of gonidia production from chromatinolysis in the "giant" cells under conditions favourable to the induction of moderate degrees of plasmolysis. And it is noteworthy that this chromatinolysis in giant-cells in meningococcal cultures is best seen in media containing serum. It is also to be noted, emphasis being laid upon this point in the case of the enteric organisms, that these unfamiliar methods of reproduction have not been noted in cultures which appear to be exclusively composed of normal populations, the ordinary vegetative forms appearing not to depart from the ordinary methods of reproduction by equal binary fission only, even when conditions are markedly favourable for unusual types of reproduction. In the case of meningococcal cultures it appears possible that the explanation of the fact that some cultures will not easily survive, whilst others readily survive, is due to the exclusive presence of vegetative forms in the former case, and in admixture with fertile giant-forms in the second case, these being able to survive adverse circumstance in consequence of increased resistance to such circumstance, as was found to be the case with organisms of the enteric group.



## CONCLUSIONS.

There appears, from these observations, to be no doubt that changes in agglutinability are often associated, in the case of the organisms examined, with the occurrence of developmental changes in such organisms, changes which have in many cases definite morphological values. It must however not be supposed that a claim is here put forward that a different morphological equivalent exists for each of the agglutination vagaries here disclosed. The subject is far too complex a one for any such claim to be made. The evidence so far produced is that of a preliminary investigation only, and is published now in the hope that other workers will devote their attention to the morphological problems dealt with. In the meanwhile an attempt is being made to correlate the results obtained with the results of testing subcultures from single cells, and to show that reliable polyvalent serums can be obtained by construing valency in terms of morphology and not in terms of "strains" unrelated to developmental phases. It will be evident that absorption tests are of little value in this work unless the morphological equivalent of each emulsion used in the preparation of a serum is first worked out.

## NOTE.

As further evidence of the reliability of the macroscopic method employed throughout these observations the subjoined figures of control titration tests may be cited, the test which gave the largest error being selected. The maximum experimental error in this test is seen to be 5.3 per cent., as gauged by titration of N/20 HCl with N/20 NaOH, each tube in the total series of 10 tubes receiving measured quantities of HCl in water as follows. First filling: equal volumes of acid in all 10 tubes. Second filling: tubes 2 to 9 receive half the volume of the preceding tube, tube 1 receiving a full volume of acid, tube 10 receiving none. Third filling: each tube receives a fresh volume of acid equal to the volumes employed in the first filling. In this way the distribution of saline, serum and emulsion was reproduced as closely as possible, with the result that a severe test of accuracy was imposed with regard to the thirty measurements employed.

In 6 of the 10 tubes 2.90 c.c. of N/20 NaOH were required for neutralisation.

„ 2	„	2.70 c.c.	„	„	„
„ 1	„	2.82 c.c.	„	„	„
„ 1	„	2.85 c.c.	„	„	„

In this control test therefore we get a mean of 2.80, a mode of 2.90, and a maximum experimental error of 5.3 per cent.

It has of course long been known that inconstant serological readings may occur when observations are made with different emulsions made from one living culture at different intervals of time, using the same serum throughout. In many of the observations recorded in the text it is shown that wide

variations may occur when a series of fractions of the same emulsion from one culture are put up at the same time against different serums specific to the organism under examination, the existence of multiple strains being usually regarded as adequate explanation of such variations, the absence of considerable experimental error being assumed. The control observations given in the text and in this note show that in the case of the variations here recorded the experimental error can be excluded.

In view of the fact demonstrated in the text that wide morphological variations may occur in living fertile descendants of a single cell, some of these variations being definitely associated with wide variations in serological equivalents in terms of one serum, it would appear that the multiple strain theory is here inadequate. And this view is confirmed by the following transitions occurring within a few hours.

At 10.0 a.m., 13. ii. 18, an emulsion was made in plus 10 broth from a 13 hour 4 per cent. glucose agar culture inoculated with a pure culture of *B. typhosus*, and a fraction of the living emulsion was tested at once (*vide A*) against Lister *B. typhosus* serum: C 105: 28. xi. 17: 1/20: 1/6000.

At 2.30 p.m. on the same day a second fraction of the same living emulsion in broth was, after incubation at 37° C. for 4½ hours, tested against the same serum from the same tube (*vide B*).

Twelve hours later a third fraction of the same living emulsion in broth was, after incubation at 37° C. for 16½ hours, tested against the same serum from the same tube (*vide C*).

In each case a reading was taken after one hour at 56° C., and again after a further eight hours at 56° C. There was no difference in the two readings in any tube.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	Control	
nil	nil	nil	nil	nil	nil	nil	nil	nil	... A
C A	C A	C A	C A	C A	nil	nil	nil	nil	... B
C A	C A	C A	C A	C A	C A	C A	C A	nil	... C

C A = complete agglutination

Morphology of A	...	exclusively coccoid
„ B	...	mixed coccoid and bacillary
„ C	...	bacillary population predominant

The transition from non-agglutinable to agglutinable, with corresponding morphological transition, is however not necessarily rapid, a more or less stable condition of non-agglutinability being sometimes met with which, in one case, lasted five days in spite of repeated subculture in broth. In such case the return of agglutinability, and of bacillary form, sometimes only takes place on subculturing to agar from broth, the inverse of the usual experience.

The work on which Part I of this paper is based was carried out by me in the laboratory of the Addington Park War Hospital. The work on which



Part II is based was undertaken in the Central Laboratory of the Kitchener Hospital, Brighton. In both cases the work was the outcome of previous work on bacterial morphology carried out by me as Director of the Constance Trotter Research Fund in a laboratory generously placed at my disposal by the Governing Body of the Lister Institute.

To my laboratory attendant, E. Hawkins, I am greatly indebted for scrupulous care in the preparation of media and other material.

#### NOTE EXPLAINING CHARTS.

In the following charts are given:

1. The serum dilutions employed, each dilution except the first being double that of the preceding dilution.

In all cases the end-points, denoting partial agglutination, are given, represented by the symbol +. The end-points of complete agglutinations, and symbols representing the numerous gradations between complete agglutination and that just visible with the aid of a hand-lens, are deliberately omitted so as not to confuse the issue.

In determining the end-points here recorded extreme care was taken, in each series, to study the gradations between each tube in a row of nine tubes in relation to the gradations between the tubes in all the rows. The symbol 0 represents complete absence of agglutination.

2. The numbers of each subculture, generally with the dates of subculture.

3. Keys giving the "genealogy" of the subcultures.

4. The particulars of each serum employed, as to source, date of preparation, dilution and reputed maximum titre.

5. Whenever possible, the morphological equivalent.

In Chart A the brackets opposite the numbers 3, 4, 5 = 6, 7, 8 = 11, 12 = 13, 14, 15, 16 = 1, 2, 3, 4, indicate that each group of numbers indicates identical fractions of one emulsion (*vide* NOTE).

In number 2, of date 11. i. 18, the final dilution of 10,240 was inadvertently omitted.

#### CHART A.

#### CONTROL OBSERVATIONS SHOWING RELIABILITY OF AGGLUTINATION TECHNIQUE EMPLOYED (*a*) *B. TYPHOSUS*, (*b*) *B. DYSENTERIAE*.

Morphological equivalent of each control emulsion "normal" bacilli.

#### *B. typhosus*.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	Control	Date	Number
+	+	+	+	+	+	+	+	0	10. i. 18	1
+	+	+	+	+	+	±	in last tube	0	11. i. 18	2
+	+	+	+	+	+	+	+	0	18. i. 18	3
+	+	+	+	+	+	+	+	0	"	4
+	+	+	+	+	+	+	+	0	"	5
+	+	+	+	+	+	+	+	0	5. ii. 18	6
+	+	+	+	+	+	+	+	0	"	7
+	+	+	+	+	+	+	+	0	"	8
+	+	+	+	+	+	+	+	0	8. ii. 18	9
+	+	+	+	+	+	+	+	0	11. ii. 18	10
+	+	+	+	+	+	+	+	0	28. ii. 18	11
+	+	+	+	+	+	+	+	0	"	12
+	+	+	+	+	+	+	+	0	1. iii. 18	13
+	+	+	+	+	+	+	+	0	"	14
+	+	+	+	+	+	+	+	0	"	15
+	+	+	+	+	+	+	+	0	"	16

Lister *B. typhosus* serum used throughout. Dilution 1/20: titre "maximum" 1/6000: dates 11. ix. 17: 28. xi. 17, etc.

CHART A—contd.

1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12,800	Control	Date	Number
+	+	+	+	+	+	...	...	0	1. XI. 18	1
+	+	+	+	+	+	...	...	0	„	2
+	+	+	+	+	+	...	...	0	„	3
+	+	+	+	+	+	...	...	0	„	4

R.A.M.C. Polyvalent serum = *B. dysenteriae* ("B. Y").

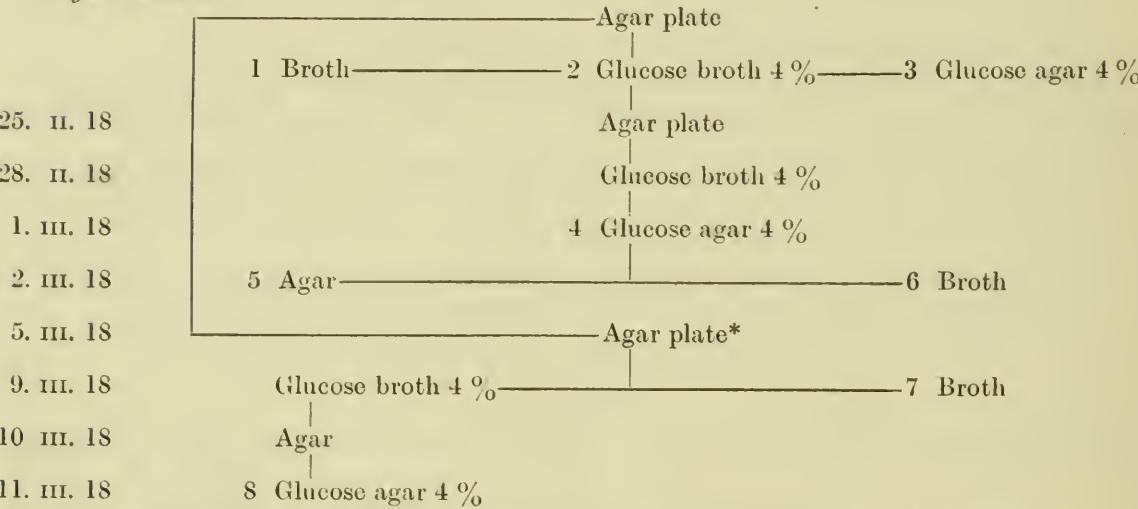
CHART 1, GROUP A.

*B. typhosus*, Strain Pierce versus Lister *B. typhosus* Serum C 105: 1/20: 1/6000: 28. XI. 17.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	Cultures	Morphology
+	+	+	+	+	+	+	+	1	Dom. pop. bacillary
+	+	+	+	+	+	+	+	2	Dom. pop. bacillary
+	+	+	+	+	+	+	0	3	Dom. pop. bacillary
0	0	0	0	0	0	0	0	4	D.P. thick-walled cocc.
+	+	+	+	+	+	+	+	5	D.P. bacillary
+	+	+	+	+	+	0	0	6	D.P. bacillary
+	+	+	+	+	+	+	+	7	D.P. bacillary
0	0	0	0	0	0	0	0	8	D.P. thick-walled cocc.

All control tubes negative.

Key to Cultures:



\* This indicates a fresh plating on 5. III. 18 direct from original agar plate.

CHART 1, GROUP B.

*B. typhosus*, Strain Pierce versus Lister *B. typhosus* Serum C 105: 1/20: 1/6000: 18. I. 18.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cult.	Morphol.
0	0	0	0	0	0	0	0	0	0	2	Coccoidal
+	+	+	0	0	0	0	0	0	0	1	Coccoidal
+	+	+	+	+	0	0	0	0	0	3	Cocco-bacill.
+	+	+	+	+	+	+	+	0	0	A	Bacillary
+	+	+	+	+	+	+	+	+	0	5	Bacillary
+	+	+	+	+	+	+	+	+	+	4	Bacillary
+	+	+	+	+	+	+	+	+	+	6	Bacillary

All control tubes negative.



Key to Cultures (Chart 1, Group B):

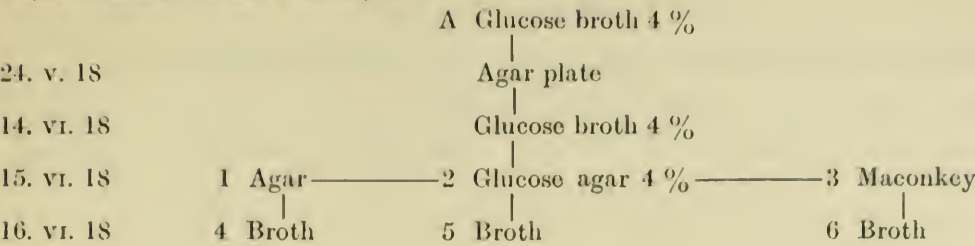


CHART 1, GROUP C.

*B. typhosus*, Strain Pierce versus Lister *B. typhosus* Serum C 105: 1/20:  
1/6000: 6. vi. 18.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cult.	Morphol.
+	+	0	0	0	0	0	0	0	0	1	Coccoidal
+	+	0	0	0	0	0	0	0	0	2	Coccoidal
+	+	+	+	+	+	+	+	+	+	3	Bacillary
0	0	0	0	0	0	0	0	0	0	4	Coccoidal
0	0	0	0	0	0	0	0	0	0	5	Coccoidal

All control tubes negative.

Key to Cultures:

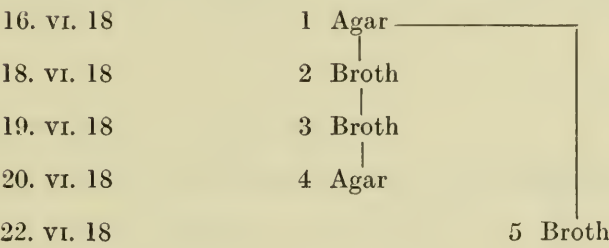


CHART 1, GROUP D.

*B. typhosus*, Strain Pierce versus Lister *B. typhosus* Serum and R.A.M.C.  
*B. typhosus* Serum.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Cultures	Serum	Colony	Cont.
+	+	+	0	0	0	0	0	0	7	Lister	A 1	0
+	+	+	+	+	+	+	0	0	5			0
+	+	+	+	+	+	+	+	0	3			0
+	+	+	+	+	+	+	+	0	1, 2, 4, 6, 8, 9, 10			0
+	+	+	0	0	0	0	0	0	7, 9	R.A.M.C.	A 2	0
+	+	+	+	0	0	0	0	0	1			0
+	+	+	+	+	0	0	0	0	2, 5, 6,			0
+	+	+	+	+	+	0	0	0	4			0
+	+	+	+	+	+	+	0	0	3, 8			0
+	+	+	+	+	+	+	+	0	10			0
+	+	0	0	0	0	0	0	0	7	Lister	B i	0
+	+	+	+	+	+	+	+	0	1, 2, 8, 9, 10			0
+	+	0	0	0	0	0	0	0	7, 9	R.A.M.C.		0
+	+	+	+	0	0	0	0	0	1			0
+	+	+	+	+	0	0	0	0	2, 10			0
+	+	+	+	+	+	0	0	0	8			0

Reproduction of Aerobic Bacteria

CHART 1, GROUP D—contd.

MAXIMUM VARIATIONS IRRESPECTIVE OF WHICH SERUM IS EMPLOYED.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Cultures	Colony
+	+	+	...	...	...	...	...	...	7, 9	A 1, A 2
+	+	+	+	...	...	...	...	...	1	
+	+	+	+	+	...	...	...	...	2, 5, 6	
+	+	+	+	+	+	...	...	...	4	
+	+	+	+	+	+	+	...	...	5, 3, 8	
+	+	+	+	+	+	+	+	...	3	
+	+	+	+	+	+	+	+	+	1, 2, 4, 6, 8, 9, 10	
+	+	...	...	...	...	...	...	...	7, 9	B 1, B 2
+	+	+	+	...	...	...	...	...	1	
+	+	+	+	+	...	...	...	...	2, 10	
+	+	+	+	+	+	...	...	...	8	
+	+	+	+	+	+	+	+	+	1, 2, 8, 9, 10	

Key to Agglutinations:

- A 1. *B. typhosus* Colony 1 v. Lister serum C 105 : 1/20 : 1/6000 : 23. VIII. 18.
- A 2. *B. typhosus* Colony 1 v. R.A.M.C. serum : 1/20 : 1/7000 : 18. IX. 18.
- B 1. *B. typhosus* Colony 1 v. Lister serum C 105 : 1/20 : 1/6000 : 23. VIII. 18.
- B 2. *B. typhosus* Colony 1 v. R.A.M.C. serum : 1/20 : 1/7000 : 18. IX. 18.

Key to Cultures:

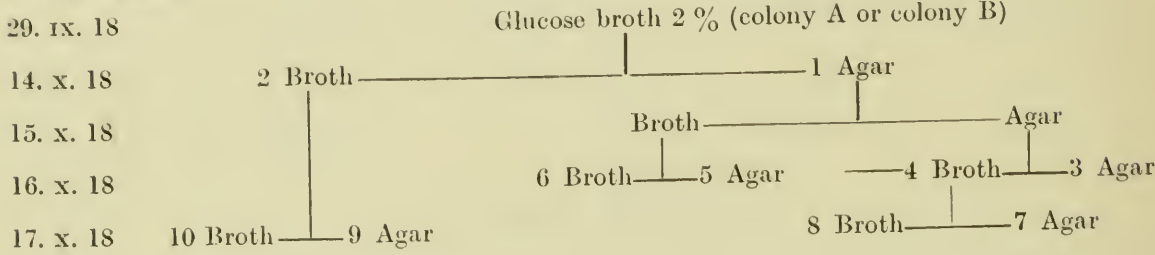


CHART 1, GROUP E.

*B. typhosus*, Strain Pierce versus R.A.M.C. *B. typhosus* Serum and Oxford *B. typhosus* Serum.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cult. Serum Cont.
+	+	+	+	+	+	+	+	+	+	1 Oxford 0
+	+	+	+	+	+	+	+	+	+	2 0
+	+	+	+	+	+	+	+	+	+	3 0
+	+	+	0	0	0	0	0	0	0	1 R.A.M.C. 0
+	+	+	+	0	0	0	0	0	0	2 0
+	+	+	+	+	+	0	0	0	0	3 0

Key to Cultures:

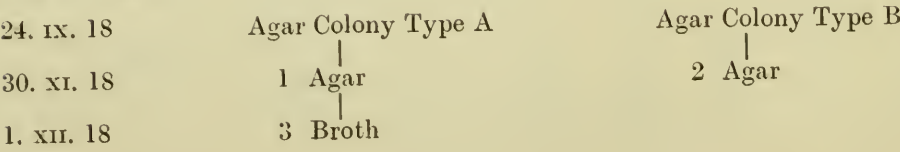




CHART 2, GROUP F.

*B. typhosus*, Oxford Strain Edwin *versus* Oxford Serum, Lister Serum and R.A.M.C. Serum.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cultures	Serum
+	+	+	+	+	0	0	0	0	0	11	Oxford
+	+	+	+	+	+	0	0	0	0	5	
+	+	+	+	+	+	+	0	0	0	4, 6, 10	
+	+	+	+	+	+	+	+	0	0	12	
+	+	+	+	+	+	+	+	0	0	5, 6, 11	Lister
+	+	+	+	+	+	+	+	+	0	4, 12	
+	+	+	+	+	+	+	+	+	0	10	
+	+	+	+	0	0	0	0	0	0	12, 6	R.A.M.C.
+	+	+	+	+	0	0	0	0	0	11, 5	
+	+	+	+	+	+	0	0	0	0	10	
+	+	+	+	+	+	+	0	0	0	4	

MAXIMUM VARIATIONS IRRESPECTIVE OF WHICH SERUM IS EMPLOYED.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cultures
+	+	+	+	...	...	...	...	...	...	12, 6
+	+	+	+	+	...	...	...	...	...	11, 5
+	+	+	+	+	+	...	...	...	...	10, 5
+	+	+	+	+	+	+	...	...	...	4, 6, 10
+	+	+	+	+	+	+	+	...	...	12, 5, 6, 11
+	+	+	+	+	+	+	+	+	...	12, 4
+	+	+	+	+	+	+	+	+	+	10

Key to Cultures:

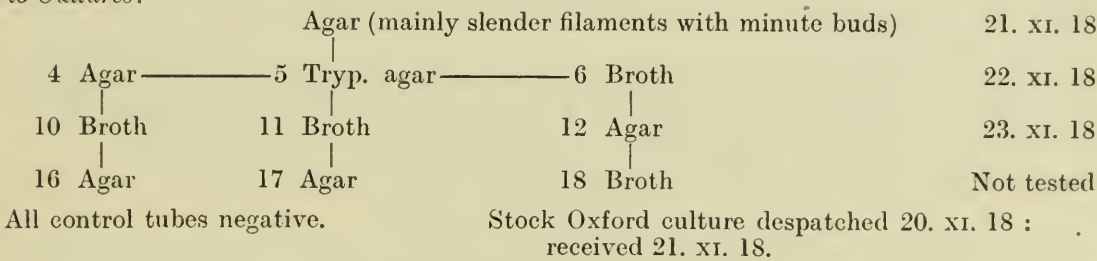


CHART 3, GROUP G.

*B. dysenteriae*: Lister Stock Flexner Culture and Lister Stock Y Culture *versus* Lister Stock Serums B. Flexner and B. Y.

E.A.M.	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control	Cultures	Experiment
10	0	0	0	0	0	0	0	0	2, 4	A
	+	+	0	0	0	0	0	0	1	
	+	+	+	0	0	0	0	0	3	
13	0	0	0	0	0	0	0	0	1, 4	B
	+	+	0	0	0	0	0	0	3	
	+	+	+	+	+	0	0	0	2	
16	0	0	0	0	0	0	0	0	2	C
	+	+	0	0	0	0	0	0	4	
	+	+	+	0	0	0	0	0	3	
	+	+	+	+	0	0	0	0	1	
13	0	0	0	0	0	0	0	0	1	D
	+	+	+	0	0	0	0	0	3, 4	
	+	+	+	+	0	0	0	0	2	

Reproduction of Aerobic Bacteria

CHART 3, GROUP G—contd.

MORPHOLOGY OF LISTER B. FLEXNER CULTURE (STOCK).

Dominant population "normal" bacilli.

MORPHOLOGY OF B. FLEXNER SUBCULTURES.

- 1. Slender bacilli, and many bacilli showing Artaud's nodes.
- 2. "Normal" bacilli.
- 3. Short fat bacilli, uniformly stained.
- 4. Small coccoids, large diplococcoids, large ovoids.

MORPHOLOGY OF LISTER B. Y CULTURE (STOCK).

Dominant population very minute cocco bacilli.

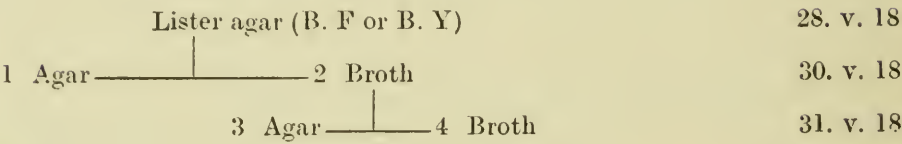
MORPHOLOGY OF B. Y SUBCULTURES.

- 1. Minute coccoids and diplococcoids.
- 2. ? "Normal" bacilli.
- 3. Minute coccoids and minute bacilli.
- 4. Minute coccoids and diplococcoids : no bacilli.

B. dysenteriae.

KEY TO CHART 3, GROUP G.

Key to Cultures:



Key to Agglutinations:

- A. Lister B. Flexner Culture v. Lister B. Flexner serum C 94 : 1/20 : 1/3000 : 27. III. 18.
- B. Lister B. Flexner Culture v. Lister B. Y serum C 137 : 1/10 : 1/1500 : 27. III. 18.
- C. Lister B. Y Culture v. Lister B. Flexner serum C 94 : 1/20 : 1/3000 : 27. III. 18.
- D. Lister B. Y Culture v. Lister B. Y serum C 137 : 1/10 : 1/1500 : 27. III. 18.

CHART 3, GROUP H.

B. dysenteriae: R.A.M.C. B. Flexner Stock Culture and R.A.M.C. B. Y Stock Culture versus R.A.M.C. Serums B. Flexner and B. Y.

E.A.M.	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control	Cultures	Experiment
15	+	+	0	0	0	0	0	0	2	A
	+	+	+	+	0	0	0	0	5, 6, 4	
	+	+	+	+	+	+	0	0	1, 3	
10	+	+	0	0	0	0	0	0	4	B
	+	+	+	0	0	0	0	0	5, 6	
	+	+	+	+	0	0	0	0	1, 2, 3	
6	0	0	0	0	0	0	0	0	2, 5, 6, 4, 3	C
	+	+	+	+	0	0	0	0	1	
17	+	+	0	0	0	0	0	0	6	D
	+	+	+	0	0	0	0	0	3	
	+	+	+	+	0	0	0	0	2, 4	
	+	+	+	+	+	0	0	0	1, 5	



CHART 3, GROUP H—*contd.*

## MORPHOLOGY OF B. FLEXNER CULTURES 3 TO 6.

3. Short bacilli.
4. "Normal" bacilli.
5. Coccoids and bacilli.
6. Short bacilli.

## MORPHOLOGY OF B. Y CULTURES 3 TO 6.

3. Bacilli greatly varying in size: large spheroidal forms.
4. Large bacilli: numerous minute bacteroids.
5. ? "Normal" bacilli.
6. Large broad bacillary forms.

*B. dysenteriae.*

## KEY TO CHART 3, GROUP H.

## Key to Cultures:

		R.A.M.C. Agar (B. F or B. Y)	24. vi. 18
1	Agar	2	Broth
5	Agar	3	Broth
4	Agar	6	Broth
			26. vi. 18
			27. vi. 18

## Key to Agglutinations:

- A. R.A.M.C. B. Flexner Culture Ledingham 24. vi. 18 v. R.A.M.C. Flexner Ledingham serum F 7 : 1/20 : 1/8000 : 27. vi. 18.
- B. R.A.M.C. B. Flexner Culture Ledingham 24. vi. 18 v. R.A.M.C. Y Ledingham serum : 1/7000 : 27. vi. 18.
- C. R.A.M.C. Y Culture Ledingham 24. vi. 18 v. R.A.M.C. Flexner Ledingham serum F 7 : 1/20 : 1/8000 : 27. vi. 18.
- D. R.A.M.C. Y Culture Ledingham 24. vi. 18 v. R.A.M.C. Y Ledingham serum : 1/7000 : 27. vi. 18.

## CHART 3, GROUP I.

*B. dysenteriae*: R.A.M.C. B. Flexner Culture Ledingham *versus* R.A.M.C.  
B. Flexner, Lister B. Flexner and Lister B. FY Serums.

E.A.M.	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Cultures	Serums	Exp.
15	+	+	0	0	0	0	0	0	0	2	R.A.M.C.	A
	+	+	+	+	0	0	0	0	0	5, 6, 4		
	+	+	+	+	+	+	0	0	0	1, 3	culture	24. vi. 18
42	+	+	0	0	0	0	0	0	0	4	R.A.M.C.	B
	+	+	+	+	+	+	0	0	0	5		
	+	+	+	+	+	+	+	+	0	1, 3	culture	2. x. 18
	+	+	+	+	+	+	+	+	+	2, 6		
5	0	0	0	0	0	0	0	0	0	1, 4, 5	Lister F C 94	
	+	+	0	0	0	0	0	0	0	3, 6		
	+	+	+	0	0	0	0	0	0	2		
32	+	+	0	0	0	0	0	0	0	4	Lister F Y C 70	
	+	+	+	+	0	0	0	0	0	5		
	+	+	+	+	+	0	0	0	0	1		
	+	+	+	+	+	+	...	...	...	3, 6		
	+	+	+	+	+	+	+	0	0	2		

# Reproduction of Aerobic Bacteria

## CHART 3, GROUP I—*contd.*

MAXIMUM VARIATIONS OBTAINED IRRESPECTIVE OF SERUMS EMPLOYED.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480
0	...	...	...	...	...	...	...	...
+	+	...	...	...	...	...	...	...
+	+	+	...	...	...	...	...	...
+	+	+	+	...	...	...	...	...
+	+	+	+	+	...	...	...	...
+	+	+	+	+	+	...	...	...
+	+	+	+	+	+	+	...	...
+	+	+	+	+	+	+	+	...
+	+	+	+	+	+	+	+	+

*B. dysenteriae.*

## KEY TO CHART 3, GROUP I.

Key to Cultures:

R.A.M.C. B. Flexner Stock Culture Ledingham				24. vi. 18	
Agar				2. x. 18	
1 Agar				2 Broth	3. x. 18
5 Agar	3 Broth	4 Agar	6 Broth	4. x. 18	

Key to Agglutinations:

- A. R.A.M.C. B. Flexner Culture Ledingham 24. vi. 18 v. R.A.M.C. B. Flexner Ledingham serum F 7: 1/20: 27. vi. 18.
- B. R.A.M.C. B. Flexner Culture Ledingham 2. x. 18 v. R.A.M.C. B. Flexner Ledingham serum F 7: 1/20: 27. vi. 18.
- C. R.A.M.C. B. Flexner Culture Ledingham 2. x. 18 v. Lister B. Flexner serum C 94: 1/20: 1/3000: 27. III. 18.
- D. R.A.M.C. B. Flexner Culture Ledingham 2. x. 18 v. Lister B. FY serum C 70: 1/10: 1/2000: 12. I. 18(?).

## CHART 3, GROUP K 1.

*B. dysenteriae:* R.A.M.C. B. Flexner Culture Ledingham *versus* R.A.M.C. Serums FY 3, Y 55, F 8 A and Lister Serum FY C 137.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Plate	Medium
+	+	0	0	0	0	0	0	0	A	G $\frac{1}{2}$ colony	Agar
+	+	+	0	0	0	0	0	0	B, C	"	"
+	+	+	+	+	+	+	+	+	D	"	"
+	+	+	+	0	0	0	0	0	B	G $\frac{1}{2}$ colony	Broth
+	+	+	+	+	+	0	0	0	C	"	"
+	+	+	+	+	+	+	0	0	A	"	"
+	+	+	+	+	+	+	+	+	D	"	"
+	0	0	0	0	0	0	0	0	B, D	H $\frac{1}{2}$ colony	Agar
+	+	+	0	0	0	0	0	0	A, C	"	"
+	+	+	+	0	0	0	0	0	A, B	H $\frac{1}{2}$ colony	Broth
+	+	+	+	+	0	0	0	0	C	"	"
+	+	+	+	+	+	+	0	0	D	"	"

(A) R.A.M.C. serum F Y 3; (B) R.A.M.C. serum Y 55; (C) Lister serum FY C 137; (D) R.A.M.C. serum F 8 A.





Reproduction of Aerobic Bacteria

CHART 3, GROUP K 3.

*B. dysenteriae*: R.A.M.C. B. Flexner Culture Ledingham *versus* R.A.M.C. Serums FY 3, Y 55, F 8 A and Lister Serum C 94.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Plate	Medium
0	0	0	0	0	0	0	0	0	A	E ½ colony	Agar
+	+	0	0	0	0	0	0	0	B	„	„
+	+	+	0	0	0	0	0	0	C	„	„
+	+	+	+	+	+	+	+	+	D	„	„
+	0	0	0	0	0	0	0	0	D	E ½ colony	Broth
+	+	+	0	0	0	0	0	0	B	„	„
+	+	+	+	0	0	0	0	0	C	„	„
+	+	+	+	+	0	0	0	0	A	„	„
+	0	0	0	0	0	0	0	0	A	F ½ colony	Agar
+	+	+	0	0	0	0	0	0	C	„	„
+	+	+	+	0	0	0	0	0	B	„	„
+	+	+	+	+	+	+	+	+	D	„	„
+	0	0	0	0	0	0	0	0	A	F ½ colony	Broth
+	+	+	0	0	0	0	0	0	B	„	„
+	+	+	+	+	0	0	0	0	C	„	„
+	+	+	+	+	+	+	+	+	D	„	„

(A) Lister B. Flexner serum C 94; (B) R.A.M.C. serum B. Y 55; (C) R.A.M.C. serum B. FY 3; (D) R.A.M.C. serum F 8 A.

MAXIMUM VARIATION IRRESPECTIVE OF SERUM.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480
+	...	...	...	...	...	...	...	...
+	+	...	...	...	...	...	...	...
+	+	+	...	...	...	...	...	...
+	+	+	+	...	...	...	...	...
+	+	+	+	+	...	...	...	...
+	+	+	+	+	+	+	+	+

CHART 4, GROUP L 1.

*B. dysenteriae*: B. Y Kitchener Strain (B) *versus* R.A.M.C. Polyvalent Serum, FY 3, Y 55, Y 4, and Lister FY C 137 Serum.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Cultures
+	+	0	0	0	0	0	0	0	R.A.M.C. FY 3	{ 18, 19 12 10, 13 11
+	+	+	0	0	0	0	0	0		
+	+	+	+	0	0	0	0	0		
+	+	+	+	+	0	0	0	0		
+	+	+	0	0	0	0	0	0	Lister FY C 137	{ 8, 9, 12 10 11 13
+	+	+	+	0	0	0	0	0		
+	+	+	+	+	0	0	0	0		
+	+	+	+	+	+	0	0	0		
+	+	0	0	0	0	0	0	0	R.A.M.C. Polyvalent	{ 20, 21 14 16 15 17
+	+	+	+	+	0	0	0	0		
+	+	+	+	+	+	0	0	0		
+	+	+	+	+	+	0	0	0		



CHART 4, GROUP L 1—*contd.*

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Cultures
+	+	+	0	0	0	0	0	0	R.A.M.C. Y 55	13
+	+	+	+	+	0	0	0	0		8, 9
+	+	+	+	+	+	0	0	0		1, 7, 12
+	+	+	+	+	+	+	0	0		3, 6
+	+	+	+	+	+	+	+	...		10, 11
+	+	+	+	+	+	+	+	+		2, 4, 5
+	+	+	0	0	0	0	0	0	R.A.M.C. Y 4	20, 21

MAXIMUM VARIATIONS IRRESPECTIVE OF SERUMS.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480
+	+	...	...	...	...	...	...	...
+	+	+	...	...	...	...	...	...
+	+	+	+	...	...	...	...	...
+	+	+	+	+	...	...	...	...
+	+	+	+	+	+	...	...	...
+	+	+	+	+	+	+	...	...
+	+	+	+	+	+	+	+	...
+	+	+	+	+	+	+	+	+

Key to Cultures:

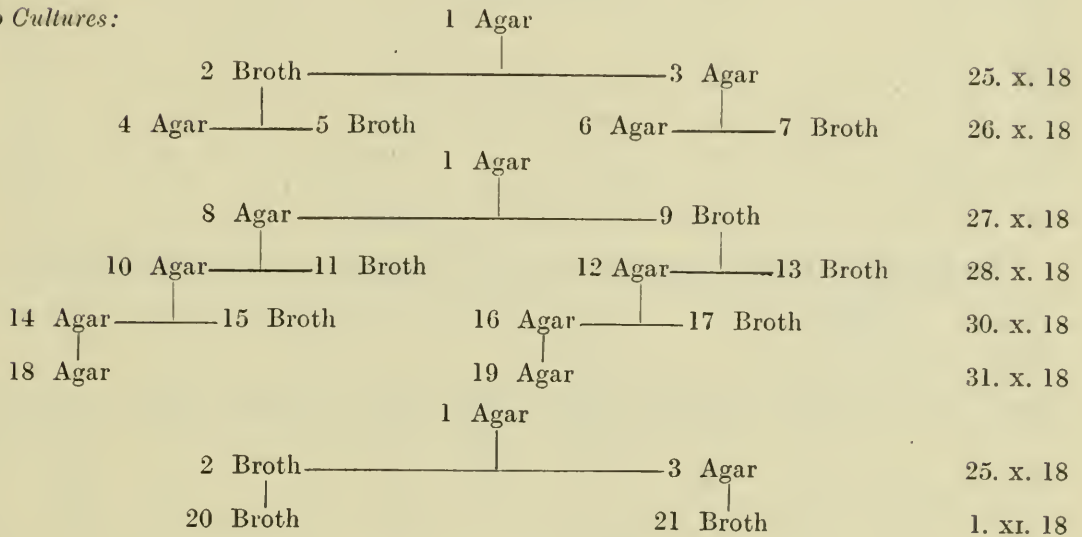
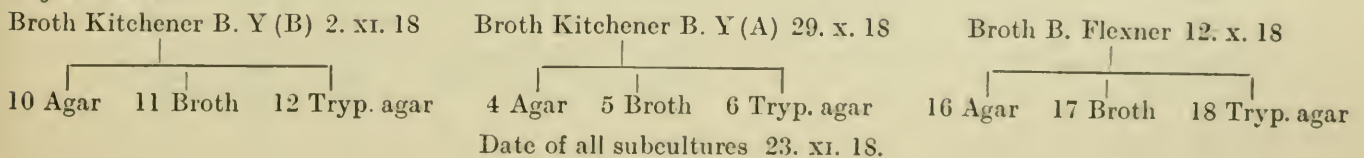


CHART 4, GROUP L 2.

*B. dysenteriae*: R.A.M.C. Polyvalent Flexner Y Serum D 1: 1/100: 1/5000:  
15. XI. 8 *versus* two Y strains and one F strain.

1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12,800	1/25,600	Cultures	Medium	Strain
+	+	+	+	+	+	0	0	0	6	Tryp. Agar	Kitchener Y (A)
+	+	+	+	+	+	+	0	0	5	Broth	
+	+	+	+	+	+	+	+	0	4	Agar	
+	+	+	+	+	+	0	0	0	10	Agar	Kitchener Y (B)
+	+	+	+	+	+	+	0	0	12	Tryp. Agar	
+	+	+	+	+	+	+	+	+	11	Broth	
+	+	+	+	0	0	0	0	0	16	Agar	R.A.M.C. B F Led.
+	+	+	+	+	0	0	0	0	18	Tryp. Agar	
+	+	+	+	+	+	0	0	0	17	Broth	

Key to Cultures:



Date of all subcultures 23. XI. 18.





CHART 5, GROUP M—*contd.*

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cultures	Serum
+	+	+	+	+	+	+	+	0	0	25	Lister
+	+	+	+	+	+	+	+	0	0	28	
+	+	+	+	+	+	+	+	+	0	27	„
+	+	+	+	+	+	+	0	0	0	31	R.A.M.C
+	+	+	+	+	+	+	0	0	0	32	„
+	+	+	+	+	+	+	+	+	0	31	Oxford
+	+	+	+	+	+	+	+	+	0	32	„

Key to Cultures:

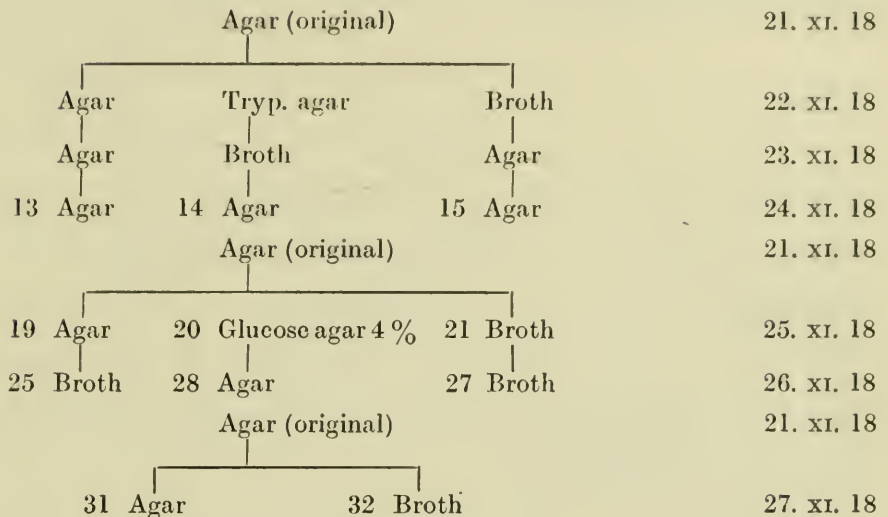


CHART 6, GROUP N.

Meningococcus Type 1 *versus* R.A.M.C. Serums Types 1, 2, 3, 4.

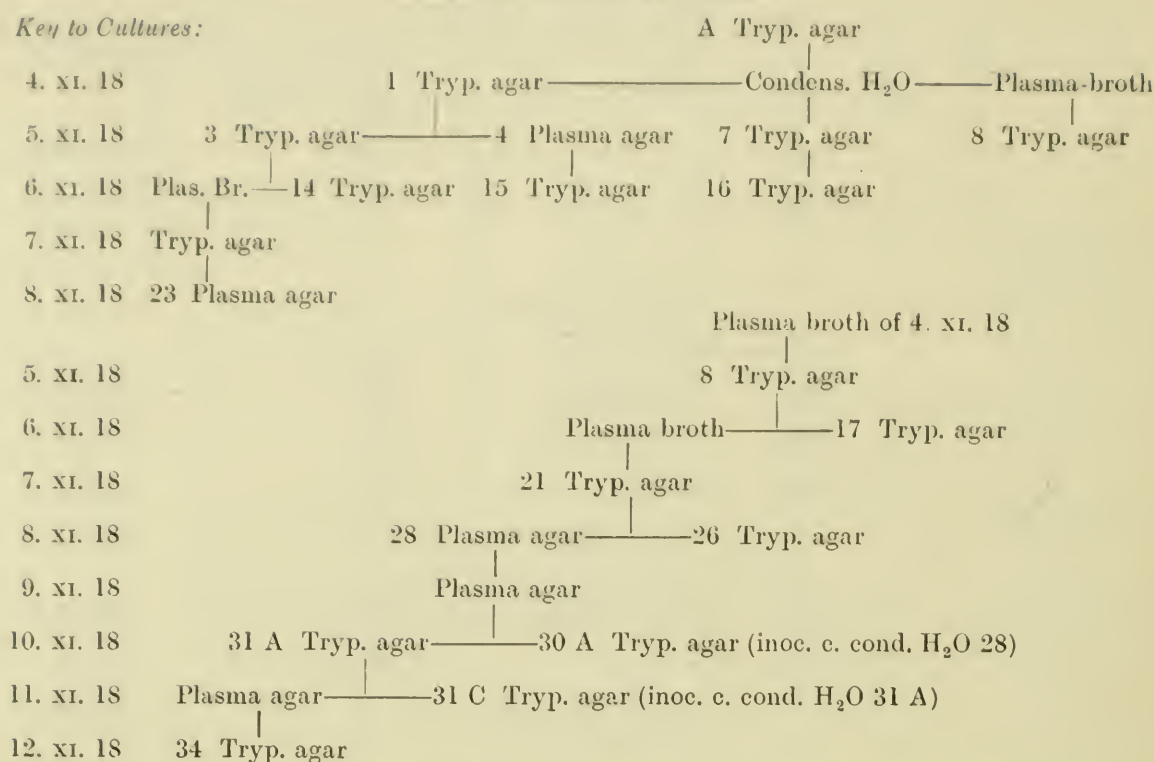
0	1/100	1/200	1/400	1/800	1/1600	Control	Cultures	Serums	Serums
+	0	0	0	0	0	0	14, 21	(J 4 E.T. 1/1000)	Type 4
+	+	0	0	0	0	0	1, 15, 16, 17, 30 A	—	—
+	+	+	0	0	0	0	3, 7, 8, 26, 31 A, 31 C, 34	—	—
+	+	+	+	0	0	0	23	—	—
+	+	+	+	+	0	0	4	—	—
+	0	0	0	0	0	0	1, 3, 4, 21	(J 3 E.T. 1/800)	Type 3
+	+	0	0	0	0	0	30 A, 34	—	—
+	+	+	0	0	0	0	7, 8, 14, 15, 26, 31 A	—	—
+	+	+	+	0	0	0	16, 17, 31 C	—	—
+	+	+	+	+	0	0	23	—	—
+	+	0	0	0	0	0	1, 3, 4, 23, 26, 30 A	(N 2 E.T. 1/800)	Type 2
+	+	+	0	0	0	0	8, 14, 15, 17, 21, 31 A, 34	—	—
+	+	+	+	0	0	0	7, 16	—	—
+	+	+	+	+	0	0	31 C	—	—
+	+	+	0	0	0	0	34, 31 C	(F 1 E.T. 1/1200)	Type 1
+	+	+	+	0	0	0	4, 7, 16, 21, 26, 30 A, 1	—	—
+	+	+	+	+	0	0	3, 8, 14, 15, 17, 23, 31 A	—	—
E.A.M. with serum type 4							17·2		
„ „ „							3 17·10		
„ „ „							2 19·12		
„ „ „							1 29·12		

Cultures 4, 23, 31 C agglutinate with serums 2, 3 or 4 respectively as well as does any single culture with serum 1: 1/800.

Cultures 23, 16, 17, 31 C, 7 agglutinate with serums 2, 3 or 4 respectively as well as do cultures 4, 7, 16, 21, 26, 30 A, 1 with serum 1: 1/400.

CHART 6, GROUP N—*contd.*

## Key to Cultures:

*Meningococcus Type 1: Morphological Equivalents of Subcultures.*

A. Chiefly small equally dividing meningococci: several deeply staining "giant" forms, some dividing equally, others unequally (budding), others showing central clear space.

1. Normal meningococci, equally dividing: uniform in size and staining: no "giant" forms seen.

3. Large non-dividing forms: three and four times the diameter of meningococci.

4. Normal meningococci mixed with minute forms, some of which are about 0.2 microns in diameter: a few small deeply-staining non-dividing forms.

7. Normal dividing meningococci, some very minute: no "giant" forms seen.

8. Normal dividing meningococci, some very minute: a few "giant" forms, numerous Gram negative minute bacilli: organisms stain uniformly.

14. *Vide* 15.

15. Medium sized "giant" forms, only a few showing signs of division: several small bacillary forms (Arkwright, d'Este Emery and others).

16. Dominant population lightly-staining "giant" forms, many undergoing unequal fission: occasional small bacillary forms.

23. Large organisms staining uniformly: many staining deeply with double contour outline, suggesting thick walls.

21. Large deeply-staining organisms, occurring singly, in short chains and in clusters. No evidence of division. Some have clear centres, in others the clear space is placed laterally, perhaps a profile effect. Population as a whole homogeneous in size. In some cases the clear centre occupies the greater part of the cell. The film does not in the least degree represent a meningococcal preparation.

26. Normal dividing meningococci, mixed in some parts of the film with large numbers of small bacilli, which are in many cases throwing off Gram negative buds, indistinguishable from meningococci: several bacilli are undergoing spherical segmentation in the terminal part of the bacillary axis.



17. No meningococci seen: population appearing entirely to consist of "giant" cells of all sizes, mainly undergoing unequal binary fission.

31 A. "Giant" cells of all sizes, from very small to large: some staining deeply, with thickened walls. A few equally dividing meningococci seen, and a few minute bacillary forms, some being of the wisp type.

30 A. Normal equally dividing meningococci, and a few large "giant" forms, with deeply-staining outline, and clear centres: numerous chains of 3, 4, 5 or 6 organisms: one chain of 8 organisms.

34. Extremely minute single and dividing cocci, some measuring about 0.1 to 0.2 microns in diameter. Some of the larger forms, from 0.4 upwards, stain more deeply than the smaller forms, some of which occur in short chains.

(Numbers refer to films and not to cultures.)

#### ROUGH SUMMARY OF RESULTS.

Although true meningococci are represented in serum 1, as shown by the high agglutination figure reached with serum 1 in the case of subculture 8, it appears that the antigenic value of strain 1, as represented in serum 1, finds its chief expression in "giant" forms, the morphological equivalent of films 3, 14, 17, 23, 31 A, from the subcultures which give the highest readings with serum 1 (except 8), being mainly that of "giant" forms in various stages of development. As I showed in 1916 "giant" forms may be either large, small or intermediate in size, and can at once be distinguished from true meningococci on the warm-stage by the fact that they often multiply by unequal binary fission, and by the further fact that they often undergo in addition endo-chromatinolysis, giving rise directly to meningococci which then divide in the ordinary vegetative manner. These fertile "giant" forms are readily distinguishable from forms undergoing genuine involutionary changes, both by the sterility of the latter when observed on the warm stage and by their feebly-staining properties.

#### REFERENCES.

- ABBOTT and GILDERSLEEVE (1904). On the branching occasionally exhibited by *B. diphtheriae*. *Centralbl. f. Bakteriolog.* xxxv. 273.
- FISCHER, A. (1900). *Zeitschr. f. Hyg.* xxxv. 58.
- HORROCKS, W. (iii. 1911). On the viability and possible variation of the *B. typhosus*. *Journ. Roy. Army Med. Corps*, 225.
- HORT, E.C. (xi. 1916). The life-histories of bacteria. *Journ. Roy. Microsc. Soc.*
- (iv. 1917). The meningococcus of Weichselbaum. *Ibid.*
- (1917). Morphological studies in the life-histories of bacteria. *Proc. Roy. Soc. B.* lxxxiii. 468.
- LEHMANN and NEUMANN (1896). *Atlas und Grundriss der Bakteriologie*.
- LÖHNIS and SMITH (31. vii. 1916). Life cycles of the bacteria. *Journ. of Agricult. Research*, 675.
- MIGULA (1900). *System der Bakteriologie*, I. 727.
- VEDDER and DUVAL (1901). The etiology of acute dysentery in the United States. *Journ. Exper. Med.* vi. 198.
- WALKER and MURRAY (vii. 1904). The effect of certain dyes upon the cultural characters of the *B. typhosus* and some other micro-organisms. *Brit. Med. Journ.* II. 17.

## EXPLANATION OF PLATES IV—VII.

## PLATE IV.

*B. typhosus* subcultured on + 10 agar from 4 % glucose broth. Composite selected fields. Agar cultures 1 to 3 hours, glucose cultures 12 hours.

1. Thick-walled cells (*a*) resting, (*b*) germinating. 2. Bacteroids living. 3. Sporangoids living. 4. Chlamydosporoids living. 5. Oidioids living. 6. Gonidioids living. 7. Coccoids stained. 8. Giant-cells stained. 9. Flagella unstained. 10. Mixed field living.

Series 1—10,  $\times 1500$ .

## PLATE V.

*B. typhosus*: warm-stage observations on + 10 agar from 4 % glucose broth.

Series A—D,  $\times 1500$ .

## PLATE VI.

*B. typhosus*: warm-stage observations on + 10 agar from 4 % glucose broth.

Series E—J,  $\times 3000$ .

## PLATE VII.

*B. typhosus*. Developmental phases associated with loss of agglutinability (photographs by F. Martin Duncan,  $\times 1000$ ).

- 1, 5. Thin-walled coccoids (agglutination nil), Lister serum.
  - 4, 8. Thick-walled coccoids and normal bacilli (agglutination 1/1280), Lister serum.
  - 2, 3, 6. Thick-walled coccoids (agglutination nil), Lister serum.
  7. Thick-walled coccoids and baccilli (agglutination 1/160), Lister serum.
- Pure bacillary subcultures from these coccoids agglutinated to 1/40,960, Lister serum. For warm-stage observations of growth from coccoid to bacillus *v.* Plate VI, G.

## TIME-TABLE OF PLATES V AND VI.

- |       |  |
|-------|--|
| A.    | 4.55, 5.10, 5.22, 5.35, 5.45, 6.0, 6.12, 6.50 p.m.         |
| B.    | 7.35, 7.50, 8.15, 8.40, 9.0, 9.20, 9.40, 10.0 p.m.         |
| C.    | 8.40, 9.10, 9.40, 10.10, 10.30 p.m.                        |
| D.    | 7.0, 7.40, 8.10, 9.0, 9.15, 9.50, 10.15 p.m.               |
| E, F. | 10.0, 10.5, 10.10, 10.20, 10.25, 10.40, 11.10 to 12.0 p.m. |
| I.    | 7.30, 8.0, 8.35, 9.0 p.m.                                  |
| J.    | 6.25, 6.35, 6.44, 6.48 p.m.                                |







1



2

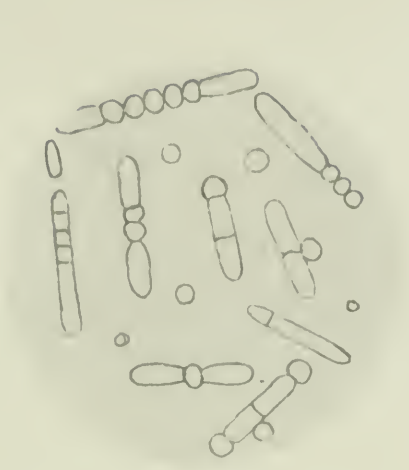


3

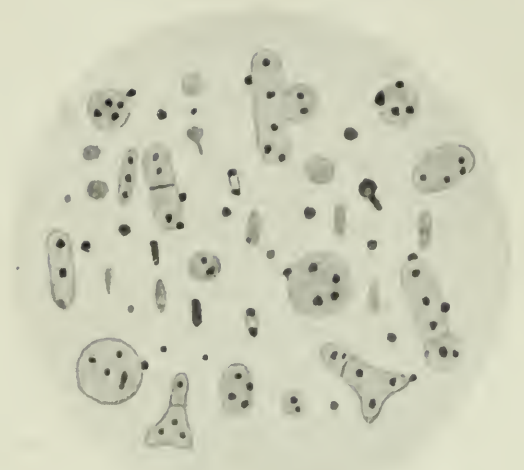


4

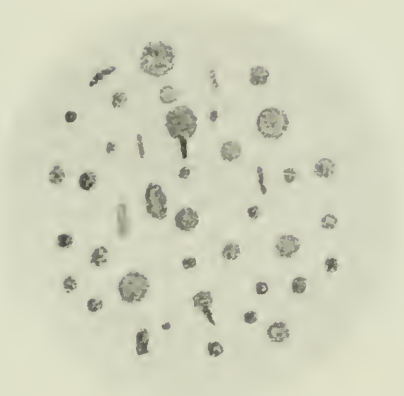




5



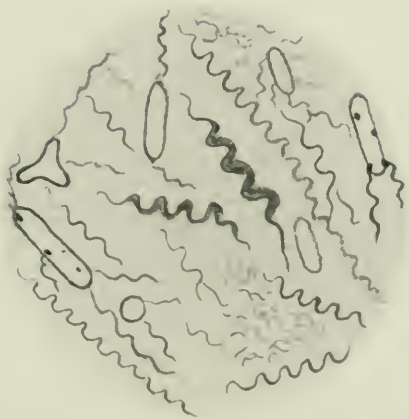
6



7



8



9



10







A

B

C

D



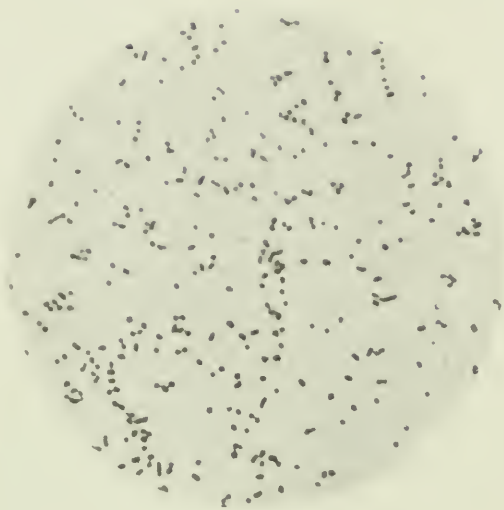




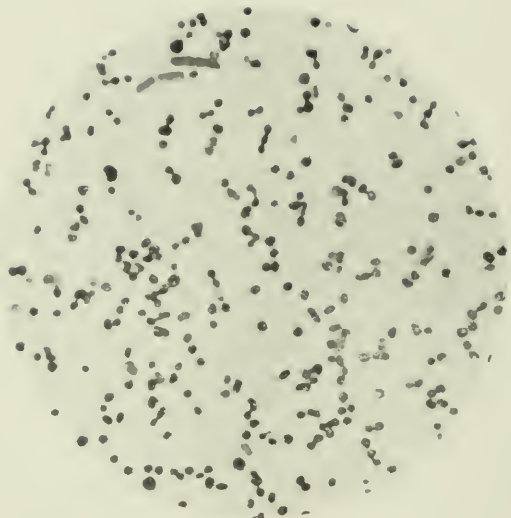




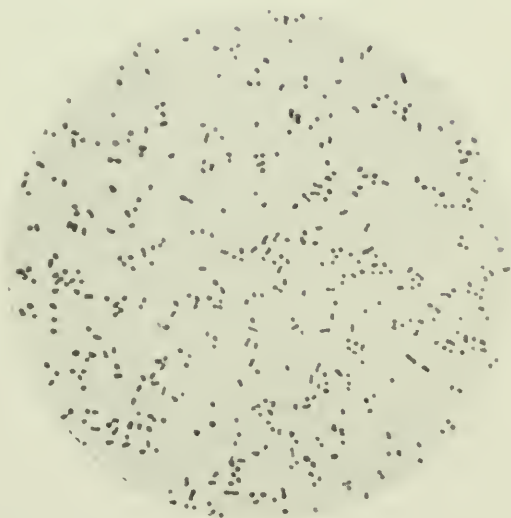




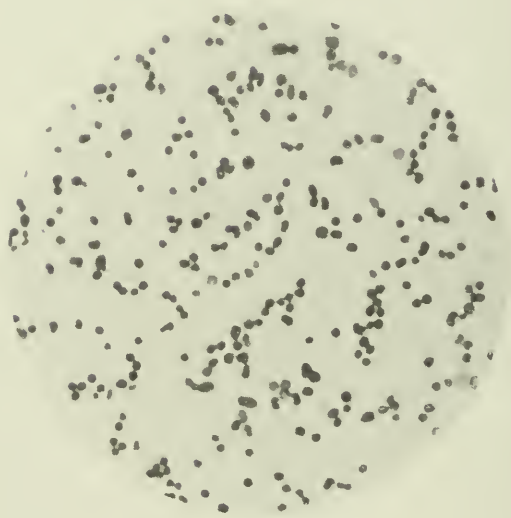
1



2



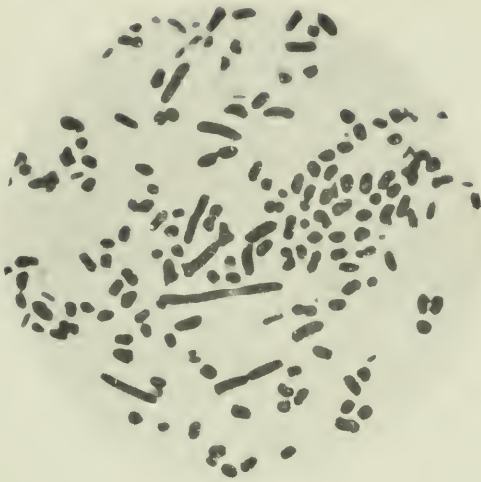
5



6



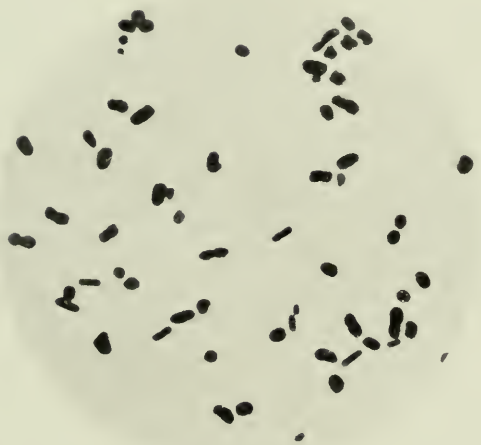
4



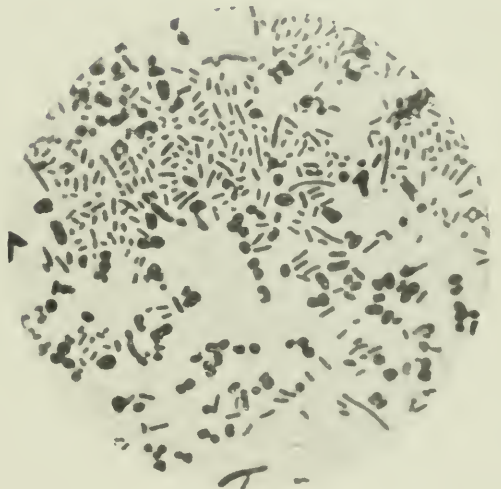
3



4



7



8





## THE DIETS OF LABOURING CLASS FAMILIES DURING THE COURSE OF THE WAR.

BY MARGARET FERGUSON, M.A.

(*From the Physiological Laboratory, University of Glasgow.*)

IN 1915-1916 I studied the dietaries of forty-seven Glasgow labouring class families with the object of comparing the food of healthy and rachitic children. The results of this work were published by the Medical Research Committee<sup>1</sup>.

In order to ascertain how the food was affected by war conditions subsequent studies were made of a few typical families at three different periods.

(1) Ten families were investigated in February 1917, when, though prices were high, all food-stuffs, save potatoes, were still plentiful.

(2) Eight of these families consented to a third study, which was carried out in November 1917, after voluntary rationing had been urged by the Minister of Food.

(3) In December 1918 five of these families were studied for a fourth time, this time during the period of compulsory rationing of meat, sugar and fats.

We have, thus, a series of four investigations of the same five families under different conditions and extending over a period of three years of the war. The present report summarises the information gathered.

The studies included three men, five women, twelve children over ten years and fourteen under ten years of age.

In each case the food was weighed for the period of a week.

The method of investigation adopted is explained in Professor Noël Paton's introduction to the report of the dietary studies made by Miss Dorothy Lindsay in 1911-1912. Miss Lindsay's work was done in Glasgow upon the same class, and forms a pre-war standard of comparison with the present results.

A comparison of the results is given in Table I.

### I. *The effect of Rationing.*

The present study shows that rationing had little effect upon the protein content or the energy value of these diets, but that the average consumption of fat fell 14 gms. per man per day. Four of the five families consumed less fat. Where strictest economy is necessary, as was the case here, the housewife generally relies on margarine as her chief source of fat, the fats in meat being

<sup>1</sup> See *Special Report Series*, No. 20, 1918, and also, from the economic standpoint, in *Proc. Roy. Soc. Edinburgh*, Vol. XXXVII, part II.

so much more expensive. Dripping and lard could have been used instead of margarine, but that would have been contrary to the usual dietary habits of the families in question, and the housewives seem to have preferred simply to reduce the allowance of fat. Table II shows the extent of the reduction.

Table I.

Energy Value, Protein and Fat Consumption per man per day calculated on the basis of Atwater's allowances.

	First study, 1915-16				Second study, Feb. 1917			
	Protein in gms.	Fat in gms.	Energy in calories	Family income	Protein in gms.	Fat in gms.	Energy in calories	Family income
S 84	86.0	93.0	2836	27s.	77.5	63.8	2530	30s.
N 31	128.9	128.2	4174	36s.	103.0	67.8	3112	39s.
H 47	88.9	67.2	3003	22s.	85.0	62.3	2714	23s. 5d.
M 112	88.0	97.5	3318	30s.	105.9	98.1	3476	55s. 6d.
N 150	148.4	105.3	3568	25s.	138.6	112.8	3690	35s. 6d.
Average	108.0	98.2	3380	—	102.0	81.0	3104	—
Average *	93.1	84.2	2897	—	87.4	69.4	2661	—
	Third study, Nov. 1917				Fourth study, Dec. 1918			
	Protein in gms.	Fat in gms.	Energy in calories	Family income	Protein in gms.	Fat in gms.	Energy in calories	Family income
S 84	82.3	66.0	2289	38s.	79.9	65.3	2713	55s.
N 31	123.1	77.2	4079	44s.	104.7	60.3	2892	41s. 6d.
H 47	100.1	77.2	3159	34s.	93.3	62.9	3003.1	34s.
M 112	119.9	134.6	3650	41s.-61s.	100.0	71.0	3332.1	61s.
N 150	105.0	92.7	3202	48s.	146.1	117.4	3691.1	68s.
Average	106.1	89.5	3276	—	104.8	75.4	3126	—
Average *	87.5	76.7	2808	—	89.6	64.6	2680	—

\* Corrected to Lusk standard (p. 411).

Table II.

Amounts consumed per man per week in lbs. on the basis of Atwater's allowances, and the food value purchased per penny spent in each study.

	First study							Second study						
	Flour	Potatoes	Meat	Sugar	Milk in c.c.s	Butter and margarine	Calories per 1d.	Flour	Potatoes	Meat	Sugar	Milk in c.c.s	Butter and margarine	Calories per 1d.
S 84	4.97	4.03	1.0	.86	2267	.51	354	5.64	.78	.91	.84	739	.64	265
N 31	7.51	1.7	1.69	2.08	2191	.52	413	7.58	.7	1.6	.68	801	.45	324
H 47	4.0	4.0	.91	1.52	868	.50	418	7.69	—	.62	.57	340	.57	330
M 112	4.55	4.52	1.71	1.43	2079	.71	400	7.87	2.1	1.79	.87	808	.85	303
N 150	7.04	8.16	3.69	.56	2464	.21	277	7.08	5.55	3.29	1.23	1360	.49	182
Av.	5.61	4.48	1.8	1.29	1974	.49	372	7.17	1.83	1.74	.84	810	.6	281
	Third study							Fourth study						
	Flour	Potatoes	Meat	Sugar	Milk in c.c.s	Butter and margarine	Calories per 1d.	Flour	Potatoes	Meat	Sugar	Milk in c.c.s	Butter and margarine	Calories per 1d.
S 84	6.26	2.04	1.18	.52	1041	.33	222	4.69	2.93	1.15	1.25	1420	.47	217
N 31	9.63	5.12	.97	1.16	2431	.88	378	7.55	3.49	1.74	.81	1453	.47	223
H 47	7.06	5.95	.96	.58	800	.57	345	6.89	3.68	.99	.83	477	.46	269
M 112	6.32	5.72	1.21	.90	1737	1.32	241	7.72	3.38	2.12	1.34	1560	.32	192
N 150	5.13	9.9	2.72	.73	1459	.58	203	6.10	5.40	4.0	.50	2470	.20	148
Av.	6.88	5.75	1.41	.78	1494	.74	278	6.59	3.78	2.0	.95	1476	.38	210



The diet which shows an increase of fat (N 150) is that of a family, which had a considerable advance of income during the war. The fat eaten by this family came mainly from meat, particularly from unrationed sausages and bacon. Indeed they did not draw their full margarine ration, and the mother was in the habit of using a small quantity of dripping each day to light the fire!

The official weekly rations at that time were: Sugar  $\frac{1}{2}$  lb., Margarine  $\frac{1}{4}$  lb. and Butter 1 oz. Meat (excluding rabbits, bacon and sausages, which were not rationed) could be purchased to the value of 1s. 3d. per person per week. There were also lard and jam rations each amounting to  $\frac{1}{4}$  lb. weekly. The former none, and the latter only two of the families purchased.

*Meat.* All the families had slightly more meat at the fourth than during previous studies. This was probably due to the butter and margarine restrictions. The average amount of rationed meat eaten during the study week was only  $\frac{1}{2}$  lb. per person.

*Sugar.* Families S 84 and M 112 had the Christmas increase to  $\frac{3}{4}$  lb. per person. N 150, on the other hand, did not use the full  $\frac{1}{2}$  lb. ration. On the whole the sugar consumed was more than at the previous study, though not so much as in 1915.

Rationing thus brought about singularly little change. The more determinative factors were income, and the dietary habits of the families.

## II. *A Consideration of the Adequacy of the Diets.*

From Table I it will be seen that, with one exception, there is a singular uniformity in the energy value of the diet of each family at the four different periods. This, however, is no guarantee that the food is sufficient. Recent investigations would seem to show that Atwater's allowances for age and sex, upon which these and all previous dietary studies have been calculated, are not adequate. Lusk has suggested that the following values may be taken as representing the ratio of the food requirements of the child to that of the average man, and this has been accepted by the Food Committee of the Royal Society, and the International Committee.

LUSK		ATWATER	
Age	Coefficients	Age	Coefficients
0- 6	0.5	Under 2	0.3
6-10	0.6	2- 5	0.4
10-13	0.83	6- 9	0.5
13-20 (boys)	1.0	10-13	0.6
Average man	1.0	14-16 (boys)	0.8
		Average man	1.0
13-20 (girls)	0.83	14-16 (girls)	0.7
Average woman	0.83	Average woman	0.8

These figures were calculated on the basis of the standard measurements of the Anthropometric Committee, 1883, and on the experimental work of Du Bois, who proved that the energy expended per unit of body surface is greater

in children than in the adult male. The adoption of Lusk's coefficients instead of Atwater's raises the average equivalent per person in the five families from 0.6 to 0.7 of a man. The values of the diets according to Lusk's allowances are given at the foot of Table I. These are much below what is usually thought necessary. The explanation of this probably lies in the fact that in three of the families the children were markedly below the Anthropometric Committee's averages. Omitting N 150, where there were only two small children under five years, whose dietary needs were small compared with those of the parents, the figures were as follows:

Height in cms.										
Age	Anthropometric Committee		N 31		S 84		H 47		M 112	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
2	85.5	81.1	—	—	—	—	66.7	—	—	—
5	104.2	103	—	—	91.4	—	—	—	—	—
6	111.8	108.9	—	—	—	—	90.1	—	—	—
7	116.8	112.9	—	—	101.0	—	—	—	108.6	—
8	119.5	118.4	—	—	—	—	—	105.4	—	—
9	126.2	123.8	126	—	—	125	—	—	111.8	—
10	131.7	129.7	132	—	—	—	—	107.6	—	—
11	135.8	134.9	—	—	131.4	—	—	—	—	—
12	139.7	141.4	—	—	—	—	123.2	—	—	125.7
13	144.6	146.7	—	144	—	—	—	125.7	—	125.7
14	150.7	151.9	—	153	138.4	—	—	—	136.5	—
15	155.4	154.6	—	156	—	—	—	—	—	—
Adult	171	159.3	—	159.2	174	161.3	170.1	156.2	—	154.7

Weight in kgms. (without clothing).										
Age	Anthropometric* Committee		N 31		S 84		H 47		M 112	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
5	18.1	17.8	—	—	14.32	—	—	—	—	—
6	20.1	18.9	—	—	—	—	14.0	—	—	—
7	22.5	21.6	—	—	15.91	—	—	—	17.27	—
8	24.9	23.6	—	—	—	—	—	19.32	—	—
9	27.4	25.2	22.05	—	—	20.45	—	—	20.0	—
10	30.6	28.1	25.45	—	—	—	—	19.54	—	—
11	32.7	30.8	—	—	30.5	—	—	—	—	—
12	34.8	34.6	—	—	—	—	25.0	—	—	23.87
13	37.5	39.5	—	31.82	—	—	—	26.82	—	23.4
14	41.7	43.9	—	44.54	30.45	—	—	—	32.5	—
15	46.7	48.3	—	47.73	—	—	—	—	—	—

\* The Anthropometric Committee's average includes clothing. In the present investigation the weight of the clothing varied from 1 kgm. in the case of children of five or six years to 3 kgm. for the older ones.

The measurements of the children in height and weight fell so far below the Anthropometric Standard upon which Lusk's coefficients are based, that an independent calculation of the probable basal needs of the various children of each family was made.

The body surface was calculated from the formula

$$S = 0.007184 \times W^{0.425} \times H^{0.725},$$



where  $S$  is the surface in square metres,  $W$  the weight in kilograms, and  $H$  the height in centimetres, and the heat evolved per square metre per hour by the formula  $\log y = 1.8362 - 0.0118x$ , where  $x$  is the age in years and  $y$  the required basal metabolism per hour.

From the total calories consumed during the week by each family were subtracted first the requirements of the parents on the basis that they were persons of average size. The women's food requirements, *i.e.* as eaten, were taken as being equal to 0.83 that of a man at a sedentary occupation, *i.e.* 2500 calories per day. Allowances were made for the men according to their occupations. The basal requirements of the children, calculated as indicated above, were then subtracted, the remainder being the energy available for movement, heat production, muscular work, digestion and growth. The Food (War) Committee of the Royal Society<sup>1</sup> have attempted to deal in this manner with the food requirements of children. Although sufficient data were not forthcoming to allow the Committee to make a definite statement, they tentatively suggest doubling the basal needs for ordinary life and trebling in the case of a very active child.

From the poor physique of the children, in the present investigation, it is apparent either that their food has not been sufficient to allow for normal growth, or that some other factor or factors have inhibited growth. In approaching this question it is necessary to find out whether the diets were the result of unrestricted choice, or whether poverty compelled the mothers to limit the children's food. Each family will be considered by itself.

*A. Families, whose diet has been fairly constant in energy value at the four periods of study.*

								Calories
S 84.	Total calories in 7 days' food	...	...	...	...	...	...	95,160
	Father, labourer, at 3500 calories daily	...	...	...	...	...	...	24,500
	Mother at 2500 calories daily	...	...	...	...	...	...	17,500
	Calories remaining for children	...	...	...	...	...	...	53,160
	Basal needs of Alexander (14)	...	...	...	...	...	...	8,701
	„ Charlie (11)	...	...	...	...	...	...	9,100
	„ Emily (9)	...	...	...	...	...	...	7,651
	„ Tommy (7)	...	...	...	...	...	...	6,713
	„ David (5)	...	...	...	...	...	...	5,999
	„ Baby (3)	...	...	...	...	...	...	5,250
	Total basal needs of children for 7 days	...	...	...	...	...	...	43,414
	Calories in the food available for energy expenditure in growth, etc.	...	...	...	...	...	...	9,746

or 22 per cent. of the basal needs.

All the children are below the Anthropometric Standard Average. Allowing 2 kgms. for clothing their average deficiency in weight is 3.7 kgms. or 13 per cent.

<sup>1</sup> *Report on the Food Requirements of Man and their Variations according to Age, Sex, Size and Occupation*, March 1919.

								Calories
H 47.	Total calories in 7 days' food	...	...	...	...	...	...	100,894
	Father, shoemaker, at 3150 calories daily	...	...	...	...	...	...	22,050
	Mother 2500 calories daily	...	...	...	...	...	...	17,500
	Remainder for children	...	...	...	...	...	...	61,344
	Basal needs of Hannah (13)	...	...	...	...	...	...	7,903
	„ Walter (12)	...	...	...	...	...	...	7,609
	„ Ina (10)	...	...	...	...	...	...	6,629
	„ Jessie (8)	...	...	...	...	...	...	6,692
	„ William (6)	...	...	...	...	...	...	5,600
	„ Sandy (2)	...	...	...	...	...	...	5,250
	Total basal needs of children for 7 days	...	...	...	...	...	...	39,683
	Calories available in the food for energy expenditure in movement, growth, etc.	...	...	...	...	...	...	21,661

or 55 per cent. of the basal needs.

All the children are below the average in size. Allowing 2 kgms. for clothing, their deficiency in weight averages 6.28 kgms. or 21.5 per cent.

								Calories
M 112.	Total calories in 7 days' food	...	...	...	...	...	...	84,968
	Father, seaman, for 1 whole day and 2 meals	...	...	...	...	...	...	5,830
	Mother 2500 calories daily	...	...	...	...	...	...	17,500
	Calories remaining for children	...	...	...	...	...	...	61,638
	Basal needs of James (14)	...	...	...	...	...	...	8,624
	„ Sarah (13)	...	...	...	...	...	...	7,553
	„ Peggy (13)	...	...	...	...	...	...	7,490
	„ Robert (9)	...	...	...	...	...	...	7,079
	„ David (7)	...	...	...	...	...	...	6,895
	„ Kathie (2)	...	...	...	...	...	...	5,250
	Total basal needs of children for 7 days	...	...	...	...	...	...	42,891
	Calories available in the food for energy expenditure in growth, movement, etc.	...	...	...	...	...	...	18,747

or 44 per cent. of the basal needs.

All the children are undersized. Allowing for clothing as above, they fall short by 8.71 kgms. or 25.5 per cent. of the Anthropometric Committee's averages.

*B. Family, whose diet has varied considerably from time to time.*

								Calories
N 31.	Total calories in 7 days' food	...	...	...	...	...	...	86,769
	Mother at 2500 calories daily	...	...	...	...	...	...	17,500
	Calories remaining for children	...	...	...	...	...	...	69,269
	Basal needs of Nettie (15)	...	...	...	...	...	...	10,881
	„ Bessie (14)	...	...	...	...	...	...	10,734
	„ Alice (13)	...	...	...	...	...	...	9,043
	„ Robert (10)	...	...	...	...	...	...	8,491
	„ John (9)	...	...	...	...	...	...	8,958
	„ Tommy (3½)	...	...	...	...	...	...	5,600
	Total basal needs of children (7 days)	...	...	...	...	...	...	53,707
	Energy in the food remaining for movement, growth, etc.	...	...	...	...	...	...	15,562

or 29 per cent. of the basal needs.

The two elder girls are above the Anthropometric Committee's average in size, the four younger children are slightly below. The marked fluctuations in energy value of the food consumed by this family were entirely due to changes



in income. When first studied they were in comfortable circumstances. At that time they were consuming, on Atwater's allowances, 4174 calories per man per day. At the second study, owing to the rise which had taken place in the cost of living without a corresponding increase in the family income, they could only afford 3112 calories per man per day. With an improvement in income, when the eldest girl left school, the value of the diet again rose to over 4000 calories, which is equivalent to more than twice the basal needs. In December, while the cost of living had further risen, the eldest girl had had to leave her work to help her mother at home. The Government Separation Allowance was then their only source of income, and this fact at once made its influence felt on the food. In spite of the temporary periods of shortage, the children appear to have suffered little interruption of growth.

Probably in all the foregoing families the metabolism is lower than it would be with a plentiful diet. This would make it possible for them to go on for some time on such small supplies. But if this is so, it will be accompanied by a lessening of the muscular activity necessary to healthy development.

### III. *The cost of living.*

The average value obtained by the five housewives was 210 calories per *ld*. This represents very economical purchasing, the diets including almost no milk, no eggs, little meat, little fish, and little fat. The energy came chiefly from bread and potatoes. Possibly the stunting of growth noticed in the children may be due to a deficiency in food containing the accessory growth-producing factors.

The problem narrows itself down to one of poverty. Only one family (N 150) could afford even the freedom of choice allowed by the rationing scheme. Assuming that children require twice as much food as their basal needs, we can calculate how many calories each mother ought to purchase in food. If divided by 210, the average number of calories purchasable per penny, this will represent the amount which, at the very least, each mother ought to spend on food. More could with advantage be spent so as to allow of more milk, eggs, etc. The following table compares the amount which, from the above calculation, should have been spent on food with the total weekly income of each family:

Table III.

No. of family	Cost of food			Weekly income		
		£	s. d.		£	s. d.
S 84		2	11 1		2	15 0
H 47		2	7 4		1	14 0
M 112*		2	3 4		3	1 0
N 31		2	9 11		2	1 6

\* Father intemperate, mother careless.

In addition to food the mother has to make her income cover the costs of rent, coal, gas, clothing, boots, cleansing, and insurance.

## SUMMARY.

1. Throughout the war the food value of the dietaries investigated with one exception showed great constancy, temporary shortage of certain commodities being compensated for by the greater use of others, especially of flour.

2. The food consumed was determined much more by the income and dietary habits of the families than by the restrictions imposed by rationing. The marked variations in the energy value of one dietary from time to time (normally a generous one) were directly caused by changes of income.

3. The children of three families were markedly below the average in height and weight. As the energy available in the food of these families only averaged 40 per cent. above their basal requirements calculated according to age and body surface, it seems probable that the interruption of growth had been caused by an insufficient supply of food.

4. A fourth family had at two periods of study an equally low intake of energy, but during the other two studies had at least 100 per cent. above the basal energy requirements. As the children were normal in development, growth was apparently unchecked by the temporary periods of food shortage.



## A POLYVALENT VACCINE IN THE TREATMENT OF BACILLARY DYSENTERY IN EAST AFRICA<sup>1</sup>.

By W. H. KAUNTZE, M.B.E., M.B., B.Sc. (LOND.),

*Pathologist, East Africa Protectorate*  
(late Captain, West African Medical Staff).

THE research on which this paper is based, was carried out between September 1916, and April 1917, while the writer was in charge of the Carrier Depot Hospital, Nairobi, British East Africa.

Dysentery was the most serious disease met with amongst the porters of the Military Labour Bureau in the early days of the East African Campaign, and was the main cause of death and invaliding even as late as the latter six months of 1916, although stringent sanitary measures had done much to diminish its incidence. Apparently at first it was thought that the disease was amoebic in type, and treatment consisted almost entirely in the administration of emetine. Dr Pirie, Government Pathologist at Nairobi, was the first to point out that this idea was wrong, and in the first eight months of 1916, he conducted a series of bacteriological examinations of the stools of 56 cases of dysentery and showed that in only two cases were amoebae found while dysentery-like bacilli were actually isolated in 35·7 per cent., the main type isolated resembling *B. Shiga*, or frequently *B. Morgan*<sup>2</sup>.

It was just at the conclusion of Dr Pirie's work in June 1916, that the writer took over charge of the Carrier Hospital at Nairobi. From that time the stools of every patient admitted to hospital for dysentery or diarrhoea, were examined by the writer for protozoa, at least twice, and often thrice, as a routine practice, and it was soon apparent from the number of negative results that the conclusions of Dr Pirie were justified, and that we were dealing with an outbreak of dysentery of the bacillary type and not of the amoebic type. This was also borne out by the failure of emetine in the treatment of the disease. As a result of this work the routine administration of emetine ceased, and reliance was placed mainly on saline treatment, though all the usually recommended lines of treatment for bacillary dysentery were tried at one time or other. Still the death rate did not improve as it should have done, and a very large number of cases became more or less chronic. At last it occurred to the writer that a vaccine based on the results of Dr Pirie's research might improve matters, and Dr Ross, the Director of Laboratories,

<sup>1</sup> A preliminary note on this work has appeared in the *British Medical Journal*.

<sup>2</sup> *Journ. of Hygiene*, xv. No. 4.

B.E.A., and Dr Pirie were consulted on the subject. They thought that the idea was worth a trial and agreed to prepare the vaccine. The main difficulty lay in the highly toxic nature of dysentery vaccines prepared in the usual manner, but it was surmounted by Dr Ross's suggestion to sterilise the proposed vaccine by 0·4 per cent. carbolic acid and not by heat. It may be as well to state here, particularly as it is apparently the first occasion on which a dysentery vaccine sterilized in this way has been used on such an extensive scale, that the result was most satisfactory, for in no single instance, either in the prophylactic inoculation of 76,000 porters with the vaccine or in the therapeutic inoculations described in this paper, was an alarming general or even local reaction seen.

The earliest vaccine tried consisted of *B. Shiga* and *B. Flexner* in equal proportions, and as it was uncertain what was the minimum dose that produced effects and what was the maximum dose that could be given with safety, an initial dose of 5 million was tentatively tried. This was cautiously increased as the vaccine showed its value. The original vaccine was brilliantly successful in certain cases and just as distinct a failure in others, although these latter were also of the bacillary type. It was therefore concluded that probably the failures were due to other strains of dysentery bacilli causing the disease in those cases, and consequently the vaccine was made more polyvalent, and finally contained eight strains, namely *B. Shiga* three strains, *B. Flexner* two strains, *B. Morgan* three strains. This was the vaccine used in the cases recorded in the present paper.

Before discussing the results of vaccine therapy in bacillary dysentery, it seems best to give a short account of the disease as seen amongst Africans, in whom it appears to be slightly different in its manifestations and course to the disease amongst Europeans.

#### CAUSES.

In addition to the usually recognized predisposing cause of dysentery, the following factors seem to play an important part in the origination of the disease among Africans.

(1) *Change of Environment.* This factor was particularly noticeable in those recruits who arrived in Nairobi from the region around Lake Victoria Nyanza, involving a train journey of some 250 miles over a water-shed 9000 feet high. In many the disease showed itself during the actual train journey, in others it only appeared after a residence of two or three days in the Carrier Depot at Nairobi. Although in this depot there were recruits from the districts of Kikuyu and Akamba as well as these Lake people the incidence of the disease was distinctly heavier among the latter, and one was forced to conclude that the men were infected before arrival in Nairobi. The foregoing conclusion received support from the fact that this difference in incidence still obtained in Mombasa, to reach which place both Nairobi district and Lake porters had the same rail journey. This therefore raises the question as to whether Lake



tribes are dysentery “carriers,” the change in environment leading to an exacerbation of the disease. The writer hopes to be able to throw further light on this suggestion in a later paper.

(2) *Change of Diet.* In the early stages of the campaign it was not sufficiently realized that a native is subject to the same laws of dietetics as an European, and that in his home an immense amount of labour is put into the preparation and thorough cooking of his meal by his wife or wives. When this was understood and a well cooked, well balanced diet provided for the porter, an immediate lessening of intestinal disorders resulted. The African, however, undoubtedly possesses intestines peculiarly liable to be attacked by inflammatory disease caused by errors in diet, resulting in diarrhoea or even dysentery.

(3) *Helminthiasis.* This is extraordinarily prevalent and, in the writer’s opinion, is a most important predisposing cause of dysentery (possibly owing to the delicacy of the African’s intestines as mentioned above) and in a paper to be published shortly he hopes to bring forward evidence to support this statement. It is sufficient here to give a summary of the helminth infections among the cases on record in this paper as obtained from the results of the examinations of stools.

Table I.

*Helminth Infections in Dysentery Patients.*

Class of case		Total cases examined	Number of species of Helminths observed												Total of infected cases	
			Negative		One		Two		Three		Four		Five			
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Effectives	...	473	42	9	204	43	140	29.5	70	15	15	3	2	0.5	431	91
Invalids	...	272	28	10	100	37	98	36	39	14	7	3	—	—	244	90
Total	...	745	70	9.5	304	40	238	32.75	109	14.5	22	3	2	0.25	675	90.5

In contrast to this, take the following table compiled from the results of autopsies on cases not suffering from dysentery.

Table II.

*Helminth Infections in Non-Dysenteric Patients.*

Class of case		Total cases examined	Number of species of Helminths observed												Total of infected cases	
			Negative		One		Two		Three		Four		Five			
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Effectives	}	260	80	31	124	48	49	19	7	2	—	—	—	—	180	69
Invalids																
Total	...	260	80	31	124	48	49	19	7	2	—	—	—	—	180	69

## PATHOLOGY.

One of the most noticeable features about Africans suffering from dysentery is the rapid emaciation which occurs in even moderately bad cases. Forty-eight hours seem sufficient to reduce a patient to a condition of skin and bone. It is a matter of regret that no actual weight statistics are available to illustrate this.

*Central Nervous System.* This usually shows no macroscopical change. In a few cases, however, congestion of the meninges is distinct and the cerebro-spinal fluid is increased in amount. Possibly this may be associated with the acute toxæmia which is always present in these cases.

*Respiratory System.* The lungs, in almost all cases, show signs of congestion and oedema, and it is worthy of note that many cases of dysentery die as the result of a terminal pneumonia. In all the patients in which pneumonia developed in this series, the sputum invariably showed typical pneumococci only. It seems probable that this is a complication of dysentery due to the lowered resistance of the patient (especially as the African seems to be extraordinarily susceptible to pneumococcal infections), and not one of the manifestations of a generalized septicaemia.

*The Circulatory System* shows little change except in a degeneration of the heart muscle which starts as cloudy swelling and in some instances ends in actual fatty degeneration of the myocardium. The heart in most cases is somewhat dilated and often contains an antemortem clot on the right side.

*The Genito-Urinary System* is apparently unaffected, a few cadavers showed congestion of the kidney, which may have had no connection with the original disease.

*The Alimentary System* shows the majority of the lesions in this disease. In the first place the mouth, in very bad cases, may be the site of a certain amount of ulceration, and in an appreciable number of the very toxæmic patients, this developed into cancrum oris, a condition which usually led to the rapid appearance of the man in the mortuary. This lesion seemed in the majority of cases to have developed in the gum in close proximity to the teeth, and spread from there. The pus from these cases showed only the usual pyogenic cocci and some bacilli, both Gram-positive and Gram-negative, but with the means at our disposal they could not be identified.

The stomach, as a rule, was normal in appearance, but in a few isolated cases, in which toxæmia was most distinct, ecchymoses were present in the mucous membrane and in the serous covering.

The small intestines presented a normal appearance, with a few exceptions in which ecchymoses were seen near the lower ends of the ileum, and occasionally slight superficial ulceration was present in the same area. Peyer's patches were not enlarged. The lymphatic glands in the mesentery were usually enlarged and sometimes showed small hæmorrhages in their substance, but no organisms could be isolated from them.



The large intestine was the site of the most extensive and characteristic lesions. As a rule these were confined to the sigmoid and descending colon, but were also found in seven cases in the ascending colon and caecum. No lesion of the appendix was ever observed.

The lesions were divisible into three main types:

Type (1). This was evidently an early form and was seen in cases which succumbed to toxæmia in the very early days of the disease, or in cases which were suffering from some other ailment and in which dysentery had developed in the later stages of that illness. The sigmoid colon and descending colon showed a dark red velvety surface, the mucous membrane being slightly thickened, and here and there small petechial hæmorrhages. The surface of the mucous membrane was covered with a layer of mucus, more or less faecal-stained. The blood vessels running to the affected portion of the large intestine were engorged as a rule and the veins in the actual walls of the intestine were in a condition of venous congestion. No signs of ulceration were present, nor were there any raised yellow nodules on the surface of the intestine suggestive of an amoebic origin of the disease. Sections of the intestine showed engorgement of the mucous cells of the glands of the large intestine, and a diapedesis of polymorpho-nuclear leucocytes into the submucous layer. In three cases a similar appearance was seen in the caecum and ascending colon, either as a concomitant of the disease in the descending colon, or as a solitary lesion.

Type (2). The second stage was evidently only an extension of the first. The lesions were found in the same portions of the intestines, but as a rule occupying a more extended area. The surface of the intestine was covered with a yellowish or dark brown slough, either continuously in the very worst cases, or in patches of varying size. On removal of this slough, an ulcer was left beneath, which, as a rule, presented an appearance of only affecting the mucous membrane and the submucous coat. The edges were irregular in outline, and perpendicular more or less to the floor, which was sometimes rough and sloughy, but usually smooth and formed of the muscular tissue of the intestinal wall. In bad cases the ulceration occasionally extended down to the surface of the serous membrane, but in none of the cases which died, amongst the ones recorded in this paper, was perforation seen. A section of the intestine showed, in the ulcerated area, the usual appearance of coagulation necrosis at the edge of the ulcers, and outside this area the appearance seen in the first stage of the disease described above.

Type (3). The third stage was only seen in chronic cases which had responded to treatment at first, inasmuch as the acute signs of dysentery ceased, but which had developed into a condition of chronic diarrhoea with emaciation. The appearances presented varied enormously. The areas of the intestine affected corresponded to those in the first and second stages. In these cases the large intestine was full of a light-coloured yellowish fluid containing nodules of solid faecal material. Very little mucus was noticeable

on the surface of the intestine. In some cases ulceration of the intestine was still present, but the ulcers were clean with puckered, thickened edges, and apparently in a state where an attempt at healing was taking place. The ulcerated areas were, as a rule, small compared to those in stage 2. The intervening mucous membrane showed scarred areas of fibrous tissue, evidently the site of former ulceration, and much thickening of the remaining unaffected surface. This latter portion showed a velvety appearance with marked rugae. Sections through the ulcers showed regeneration changes in the edges, but, as a rule, little or none in the floor of the ulcer which was practically always formed of the muscular coat.

In other cases no actual ulceration was present, but large areas of the large intestine showed a smooth glazed surface which were the sites of former ulcers, and between these, thickened rugose mucous membrane with markedly velvety appearance. In these cases death was apparently due to loss of fluid owing to a large portion of the large intestine having been thrown out of operation as an absorbing area.

The liver in acute cases was enlarged and engorged. No abscess was seen in it, except in one case which was suffering from *Schistosomum mansoni*, and a scraping of the walls of the abscess showed the eggs of this helminth, but no amoebae.

The spleen in acute cases was enlarged and soft. In chronic cases it was also usually enlarged, but whether this was due to dysentery or to chronic malaria, it would be difficult to say.

The other organs in the alimentary system were normal.

#### SYMPTOMS.

In an African native the symptoms usually associated with dysentery are not so distinct as in the case of Europeans, either from the native dislike of hospital treatment, or from the undoubted fact that, taken as a whole, he is less sensible to pain. The writer personally has seen cases which have gone out to duty involving hard manual labour while suffering with severe dysentery of several days standing, and which, on being called out as suspected cases, have indignantly denied being ill. The consequence of this is that, in a large proportion of cases, men have not come for treatment till the disease is well advanced, and a toxæmic condition already manifest. Dysentery is not usually a disease simulated by the malingerer, owing to the native's dislike of light diet. As a rule the patient only complains of passing blood in the stools, and diarrhoea. On further questioning he may confess to having a certain amount of pain in the stomach and straining at stool. Headache and fever are also complained of in a small proportion of cases.



## PHYSICAL SIGNS.

As has been noted above, the patient often shows signs of the onset of toxæmia in wasting, lethargy, and general weakness. The rapidity with which a native wastes as the result of disease, particularly abdominal disease, is remarkable, and is only equalled by the rapidity with which he puts on flesh once a cure is effected and good diet instituted.

The temperature varies considerably. Some cases never show any rise of temperature from the beginning to the close of the illness, others show an intermittent temperature running up to  $101^{\circ}$  F. throughout the whole course of the disease, the majority show an initial rise of temperature from  $100^{\circ}$  F. to  $102^{\circ}$  F., which, with remissions, lasts for two to three days. A subnormal temperature is usually noticeable before death.

The pulse and respiration rates closely follow the temperature, but as a rule are slightly more rapid than normal during apyrexia, rising to about 80 and 30 respectively.

The tongue is covered with a brownish white fur, but in cases showing distinct toxæmia, it becomes shrunken, covered with brown fur, and is moved with difficulty. The teeth and lips in this toxæmic stage are covered with sordes.

The abdomen as a rule presents no abnormal appearance, though if the disease has been in existence some days, it may be sunken. On palpation, a tender area in the left iliac fossa is discernible. The area of tenderness varies in extent with the severity of the disease, but is usually most marked in the region of the sigmoid colon, and diminishes as one palpates along the length of the descending colon to the splenic flexure. In very bad cases, the colon may be tender and thickened along its whole length.

The stool varies greatly in appearance. The amount of blood may be almost infinitesimal, and in some cases requires a microscope for its detection, or it may constitute the major portion of the evacuation. Mucus is always found if searched for, and usually in fairly large quantities. It is not mixed intimately with the blood, the latter forming streaks on its surface. The actual faecal content of the stool in the early stages of the disease is small, but increases with the administration of purgatives. It is rarely normal in colour, being either paler than usual and even in some cases quite white, or else green. In either case the smell indicates an increase in intestinal fermentation gases. In the very early stages of the disease the stool may be quite formed, the mucus and blood forming a coating for more or less scybalous masses, but this rapidly disappears, and diarrhoea becomes a constant feature. It is very noticeable that the number of stools rarely exceeds 20 a day and is usually not more than 10 to 15.

The liver nearly always shows an increase in size, to about a finger's breadth below the costal margin, and may be slightly tender to the touch. The spleen is rarely affected.

The respiratory system shows no physical sign, unless death is supervening, when indications of hypostatic congestion are found, and in certain cases a terminal lobar pneumonia may end the scene.

The genito-urinary system is not as a rule affected. The urine is usually highly acid, may be small in quantity and consequently highly coloured. Although it was never actually determined, the writer is inclined to believe that the colouring matter is actually increased and not relatively so as the result of concentration.

The blood shows a slight decrease in the content of red blood corpuscles, and a slight polymorphonuclear leucocytosis.

In cases in which toxaemia is marked, the face becomes sunken, the lips and teeth covered with sordes, the tongue covered with brown fur, the abdomen boat-shaped, the patient restless and semi-conscious or delirious. Hiccough then starts, and is nearly always a sign that death is near. In only a few cases when hiccough once sets in, has recovery occurred. In certain patients, hiccough was the first symptom of the onset of toxaemia.

The circulatory system does not, as a rule, show any change. In a few cases when the disease had lasted some considerable time, or toxaemia was well marked, haemic murmurs appeared, but very little dilatation of the heart was observable antemortem.

#### COMPLICATIONS.

(1) *Cancerum Oris*. This occurred in a few cases in which the dysentery had lasted for some time, and the patient was in a state of chronic toxaemia.

(2) *Perforation*. No case of perforation was ever seen.

(3) *Peritonitis*. No case with peritonitis occurred among these cases.

(4) *Toxaemia*. This is a very frequent complication of bacillary dysentery. Owing to the pressure of routine work, it was impossible to estimate the acidity of the blood in these cases, but it seems probable that an acidosis was responsible for these symptoms. It is hoped in a later paper to elucidate this point. At least it can be recorded here that alkaline treatment gave promise of better results than any other in combating this complication.

(5) *Tissue Desiccation*. This is a frequent cause of death in bacillary dysentery, the constant loss of fluid by the bowel causing a corresponding loss of water by the body cells. Although everything was done to increase the quantity of fluid taken by the patient, it was difficult to overcome the African's rooted idea that the drinking of water leads to increased diarrhoea. Objections were even raised by the natives to fluid diet on this score. Recourse to subcutaneous saline infusion was consequently often necessary.

(6) *Post-Dysenteric Diarrhoea*. Frequently after all signs of dysentery have ceased, an intractable diarrhoea persists, often accompanied by marked toxaemia and wasting. The most probable explanation seems to be that a secondary infection of the ulcers in the large intestine with *B. coli communis* or other intestinal organisms takes place.



## TREATMENT.

Practically every recommended method of treatment was tried during the time the writer was in charge of the hospital. Before his advent, emetine was given to all cases with any symptoms of dysentery, and as far as could be seen from clinical records, without success of any sort. The routine dose was one grain hypodermically once daily for seven to ten doses. Inasmuch as the clinical benefits were negative, and no evidence of amoebic infection was found microscopically or postmortem, the administration of emetine was stopped, except in cases in which amoebae were found by microscopic examination.

Small doses of calomel namely half a grain every hour for eight hours daily for three days, was a form of drug treatment largely used in the earlier cases of this series. Theoretically the idea of stimulating the secretion of bile seems to be an excellent one, inasmuch, as has been mentioned above, the biliary function of the liver seems disorganized in bacillary dysentery, as shown by the green or light coloured stools so frequently seen. Practically it was found that this treatment was too drastic for the majority of the patients suffering from this disease and eventually it was abandoned.

The sulphates of magnesium and sodium were next tried, and it was found that better results were secured by the former salt. Although it is said to exercise a specific effect in bacillary dysentery, this was found not to be the case, as alone without vaccine treatment, it had hardly any perceptible effect on the course of the disease. Combined with vaccine treatment, it was however excellent, and consequently it remained the main drug in the oral treatment of the disease. It may be remarked here that it seems strange that sodium sulphate, which is believed to act more strongly on the biliary secretion than magnesium sulphate, should have a weaker therapeutic action than the latter, but such is the practical experience in this series of cases. Other drugs tried for oral treatment were *mistura chlorinata* consisting of potassium chlorate  $2\frac{1}{2}$  grains, acid hydrochloric pure  $3\frac{1}{2}$  minims, quinine hydrochloride 3 grains, syrup of lemon 40 minims, water to 1 ounce, given every four hours, this being without any appreciable effect on the disease, and castor oil in 4 drachm doses thrice daily also with results distinctly inferior to those of magnesium sulphate.

In addition to these medicines, the object of which was either to wash out the toxins from the bowel or to inhibit bacterial development in the intestines, other drugs were used. Thus, as a result of the discovery of the vast number of dysentery patients suffering from worms (one patient passed a *Taenia saginata* which was rolled into a ball the size of a football, took 20 minutes to pass, and when laid out was 250 feet long and only one head was found in the whole mass; another passed 80 *Ascaris lumbricoides*, 30 on the first day and 50 on the second), anti-helminthics were always administered directly a patient entered hospital, and repeated at intervals till microscopic examination showed that the stools were negative. Of the anti-helminthics, thymol was found to

be the best, given in three doses of 30 grains at intervals of 2 hours on an empty stomach, followed by a dose of magnesium sulphate. Of the others, beta-naphthol was found the second best, while santonin proved the most effective in the treatment of ascariasis. Thymol was equally effective with tapeworms as with ankylostomes, surpassing Felix mas in the elimination of the former infection.

At a late stage of this series, the writer's attention was drawn to a note in the *British Medical Journal* relating to the use of Tinctura Rhei Composita in post-dysenteric diarrhoea. This drug was tried in three cases, and found so efficient in 4 drachm doses repeated at intervals of four days when necessary, that it became a routine treatment, and in at least two cases of children suffering from dysentery, it was found to effect a cure quite apart from other treatment.

As mechanical methods for cleaning the inflamed bowel and diminishing toxin absorption, rectal irrigations of sodium carbonate, potassium permanganate, tannic acid, silver nitrate, and protargol were all tried in succession, but it must be confessed without any improvement in the case. It seems probable that the injection, inasmuch as it could only be given by native orderlies, failed to reach the inflamed portion of the bowel, and was so disliked by the African that the mental depression it produced probably more than counteracted any good done by the actual irrigation.

As regards complications tissue desiccation was met by enforcing the taking of fluid by mouth, and when this was not enough, by subcutaneous saline infusions. In the later cases of the series, when the condition of the patients was extremely critical even when admitted, the latter treatment was called for from the first, and in many cases undoubtedly saved the patient's life. In the light of later knowledge, however, the writer would use an isotonic solution of sodium bicarbonate, and would be tempted to try Professor Bayliss's 6 per cent. gum arabic in this solution. The infusion acts partly by replacing lost tissue fluid, partly by raising the blood pressure and partly by diluting the toxins in the blood.

Toxaemia was combated by the administration of potassium bicarbonate in 30 gr. doses four times daily, and when the potassium salt was not available, that of sodium. This was based on a supposition yet unproved that the toxæmia was due to acidosis. Certainly in some cases it did good, and was one of the most effective drugs in relieving hiccough, which again was probably only a result of acidosis. As a further attempt to diminish the absorption of toxins from the bowel, caecostomy was performed in two cases and continuous saline irrigation instituted. Unfortunately though both cases survived the actual operation, it was apparently performed at too late a stage of the disease, and both succumbed to toxæmia. The main difficulty in this treatment seems to be to select cases late enough to justify the performance of the operation, yet early enough to give the patient a reasonable hope of recovery. Still it seems better to operate too early than too late.



For hiccough, potassium bromide in 10 gr. doses thrice daily, and a mustard plaster on the epigastrium seemed to be the most effective measures of treatment. Tinctura iodi in one minim doses in water every hour for six hours was very useful in certain obstinate cases.

Cancrum Oris was always fatal until it was treated by a potassium chlorate gargle combined with the internal administration of 15 gr. of potassium chlorate thrice daily. Under this treatment about 50 per cent. of the cases recovered.

Post-dysenteric diarrhoea was treated with considerable success by giving a vaccine of *B. coli communis* (2000 million per c.c.) in doses of  $\frac{1}{2}$  c.c., 1 c.c., and 2 c.c. at intervals of three days, Tinctura Rhei Composita in 4 drachm doses at intervals of four days was also useful in certain cases.

## DIET.

Copies of two of the latest hospital dietaries are attached. In these dietaries Diet B combines the Fluid Diet and Semi-solid Diet of the dietary in force when the cases referred to in this paper were in hospital.

## DIET I.

DIET A			DIET B		
(Green Ticket)		Hour	(Yellow Ticket)		Cooking instructions
Milk	8 oz.	2 a.m.	Milk	10 oz.	<i>Milk.</i> All milk must be boiled and served to the patients <i>warm</i> .
Brandy	$\frac{1}{2}$ "	4 a.m.	Sugar	1 "	
Sugar	$\frac{1}{2}$ "	5 a.m.	Milk	10 oz.	
			Sugar	1 "	<i>Arrowroot.</i> This must be boiled with milk and sugar for half an hour.
Tea	1 pint	6 a.m.	Tea	1 pint	
Sugar	1 oz.		Sugar	1 oz.	
Milk	4 "		Milk	2 "	<i>Matama Uji.</i> This must be boiled for two hours, forming a thin gruel.
Arrowroot	4 oz.	8 a.m.	Matama uji	6 oz.	
Milk	2 "				
Sugar	$\frac{1}{2}$ "				<i>Rice Jelly.</i> The rice must be cooked for at least three hours, or longer if the rice has not reached the stage of jellification.
Milk	4 oz.	10 a.m.	Milk	4 oz.	
Brandy	$\frac{1}{2}$ "		Sugar	$\frac{1}{2}$ "	
Soup	1 pint	12 noon	Rice jelly	8 oz.	
Bread	4 oz.		Milk	4 "	
Milk	4 oz.	2 p.m.			
Brandy	$\frac{1}{2}$ "				
Tea	1 pint	4 p.m.	Tea	1 pint	
Sugar	1 oz.		Sugar	1 oz.	
Milk	4 "		Milk	2 "	
Arrowroot	4 oz.	5 p.m.	Matama uji	6 oz.	
Milk	2 "				
Sugar	$\frac{1}{2}$ "				
		6 p.m.			
Milk	8 oz.	8 p.m.			
Brandy	$\frac{1}{2}$ "	10 p.m.	Milk	10 oz.	
Sugar	$\frac{1}{2}$ "		Sugar	1 "	
		12 midnight			

Uji is a thin gruel.

Ugali is a very thick porridge.

*Bacillary Dysentery*DIET I (*cont.*).

## DIET C. Full (White Ticket).

Monday, Wednesday, Friday, and Sunday.

Diet per man		Cooking pots per 500 men	Hour	Cooking instructions
		3 of 25 gals.	2 a.m.	Boil water.
			3 a.m.	Put in crushed mealies 12 oz. per man and 1 lb. salt per 25 gallon pot. Boil fiercely till 10 a.m.
		2 of 25 gals.	4 a.m.	Put 4 oz. beans per man and $\frac{3}{4}$ lb. salt per 25 gallon pot into cold water, bring to boil and boil hard till 11 a.m.
Tea Sugar	1 pint 2 oz.	1 of 100 gals.	5.30 a.m.	Boil water, make tea 1 pint per man with 2 oz. sugar per man. Serve at 6 a.m.
		2 of 25 gals. (matama)	6 a.m.	Put 6 oz. matama flour per man into some boiling water without salt and boil till 8 a.m. making uji.
		5 of 5 gals. (rice)		Put 2 oz. per man of rice into cold water with 1 oz. salt per 5 gallon dixie and boil till 7.30 a.m., then evaporate off water over slow fire.
Matama Rice	6 oz. 2 "		8 a.m.	Serve matama uji with rice.
		1 of 25 gals. (meat)	9 a.m.	Put meat $\frac{1}{2}$ lb. per man with 1 lb. salt per 25 gallon pot into some boiling water.
		1 of 25 gals. (bones)		Put bones from this meat into cold water and boil up.
				Boil meat and bones till 12 noon, mix soup from each, and give 1 pint per man.
			10 a.m.	Pour off water from crushed mealies, transfer latter to small dixies, evaporate off remaining water over slow fire.
		2 of 25 gals.	10.30 a.m.	Put peeled bananas $\frac{1}{2}$ lb. per man with $\frac{1}{2}$ lb. salt per 25 gallon pot into boiling water, boil till 11.30 a.m., pour off water and beat bananas into a mash.
Bananas Mealies Beans Meat Soup	$\frac{1}{2}$ lb. $\frac{3}{4}$ " $\frac{1}{4}$ " $\frac{1}{2}$ " 1 pint	2 of 25 gals.	11.30 a.m.	Pour off water from beans, put latter in small dixies over a slow fire to evaporate off rest of water.
				12 noon
			3 p.m.	Put washed unpeeled sweet potatoes 3 lb. per man without salt into cold water, boil till 4.30 p.m., then pour off water and steam over a slow fire.
Sweet potatoes 3 lb.			5 p.m.	Serve sweet potatoes.

*Alternatives.*

1. Matama flour, mwele flour, wimbe flour and mohogo meal are all interchangeable when made into uji.

2. Crushed mealies can be replaced by mealie meal ugali. For this mealie meal need only be boiled for five hours but should be treated exactly the same as crushed mealies otherwise.

3. Sweet potatoes may be replaced by ordinary potatoes 2 lb.



DIET I (*cont.*).

DIET C. Full (White Ticket).

Tuesday, Thursday and Saturday.

Diet per man		Cooking pots per 500 men	Hour	Cooking instructions
Tea	1 pint }	1 of 100 gals.	5.30 a.m.	Boil water, make tea 1 pint per man with 2 oz. sugar per man. Serve at 6 a.m.
Sugar	2 oz. }	2 of 25 gals. (matama)		Put 6 oz. matama flour per man in some boiling water without salt and boil till 8 a.m. making uji.
		5 of 5 gals. (rice)		Put 2 oz. of rice per man in cold water with 1 oz. of salt per 5 gallon dixie and boil till 7.30 a.m., then evaporate off water over slow fire.
Matama uji	6 oz. }	2 of 25 gals.	8 a.m.	Serve matama uji with the rice.
Rice	2 „ }		„	Put crushed mealies 6 oz. per man in boiling water with 1 lb. salt per 25 gallon pot. Boil hard till 3 p.m.
		1 of 25 gals.	8.30 a.m.	Put 2 oz. beans per man and $\frac{3}{4}$ lb. salt per 25 gallon pot into cold water, bring to boil and boil hard till 4 p.m.
		1 of 25 gals. (meat)	9 a.m.	Put meat $\frac{1}{2}$ lb. per man with 1 lb. salt per 25 gallon pot into boiling water.
		1 of 25 gals. (bones)		Put bones from this meat into cold water and boil up. Boil meat and bones till 12 noon, mix soup from each and give 1 pint per man.
		22 of 5 gals. (dixies)	10 a.m.	Put rice 12 oz. per man into boiling water in dixies. Boil and stir well till 11.30 a.m. Add 1 oz. salt per dixie and $\frac{1}{2}$ oz. ghee per man at 10.30 a.m.
			11.30 a.m.	Put rice over small fire to evaporate off water.
Rice	12 oz. }			
Ghee	$\frac{1}{2}$ „ }		12 noon	Serve rice, meat and soup.
Meat	$\frac{1}{2}$ lb. }			
Soup	1 pint }		3 p.m.	Pour off water from crushed mealies, transfer latter to small dixies and evaporate off remaining water over slow fire.
		1 of 25 gals.	3.30 p.m.	Put peeled bananas $\frac{1}{4}$ lb. per man with $\frac{1}{2}$ lb. salt per 25 gallon pot into boiling water, boil till 4.30 p.m., pour off water and beat into mash.
			4 p.m.	Pour off water from beans, put latter into dixies over a slow fire to evaporate off remaining water.
			4.30 p.m.	Mix beans, crushed mealies and banana mash into a thick paste.
Bananas	$\frac{1}{4}$ lb. }		5 p.m.	Serve the mixed beans and mealies.
Mealies	6 oz. }			
Beans	2 oz. }			

*Alternatives.*

1. Matama flour, mwele flour, wimbe flour and mohogo meal are all interchangeable when made into uji.

2. Crushed mealies can be replaced by mealie meal ugali. For this mealie meal need only be boiled for five hours but should be treated exactly the same as crushed mealies otherwise.

DIET II.

DIET A			DIET B		
(Green Ticket)		Hour	(Yellow Ticket)		Cooking instructions
Milk	8 oz.	2 a.m.	Milk	10 oz.	All milk must be boiled and served to the patient <i>warm</i> .
Brandy	$\frac{1}{2}$ „		Sugar	1 „	
Sugar	$\frac{1}{2}$ „				
Milk	8 oz.	4 a.m.			<i>Arrowroot</i> . This must be boiled with milk and sugar for half an hour.
Brandy	$\frac{1}{2}$ „				
Sugar	$\frac{1}{2}$ „				
Milk	8 oz.	5 a.m.	Milk	10 oz.	4 ounces making 2 pints.
Brandy	$\frac{1}{2}$ „		Sugar	1 „	
Sugar	$\frac{1}{2}$ „				
Tea	1 pint	6 a.m.	Tea	1 pint	<i>Mealie meal uji</i> . This must be boiled for two hours forming a thin gruel.
Sugar	1 oz.		Sugar	1 oz.	
Milk	4 „		Milk	2 „	
Arrowroot	4 oz.	8 a.m.	Mealie meal uji	6 oz.	
Milk	2 „		Sugar	1 „	
Sugar	$\frac{1}{2}$ „				
Milk	4 oz.	10 a.m.	Milk	4 oz.	<i>Rice Jelly</i> . The rice must be cooked for at least three hours or longer if the rice has not reached the stage of jellification.
Brandy	$\frac{1}{2}$ „		Sugar	$\frac{1}{2}$ „	
Soup	1 pint				
Bread	4 oz.	12 noon	Milk	4 oz.	
			Rice jelly	8 „	
Milk	4 oz.	2 p.m.			
Brandy	$\frac{1}{2}$ „				
Tea	1 pint	4 p.m.	Tea	1 pint	
Sugar	1 oz.		Sugar	1 oz.	
Milk	4 „		Milk	2 „	
Arrowroot	4 oz.	5 p.m.	Mealie meal uji	6 oz.	
Milk	2 „		Sugar	1 „	
Milk	8 oz.	6 p.m.			
Brandy	$\frac{1}{2}$ „				
Sugar	$\frac{1}{2}$ „				
Milk	8 oz.	8 p.m.			
Brandy	$\frac{1}{2}$ „				
Sugar	$\frac{1}{2}$ „				
Milk	8 oz.	10 p.m.	Milk	10 oz.	
Brandy	$\frac{1}{2}$ „		Sugar	1 „	
Sugar	$\frac{1}{2}$ „				
Milk	8 oz.	12 midnight			
Brandy	$\frac{1}{2}$ „				
Sugar	$\frac{1}{2}$ „				

Uji is a thin gruel.  
Ugali is a very thick porridge.

DIET C. (White Ticket.)

Diet per man		Cooking pots per 500 men	Hour	Cooking instructions
Tea	1 pint	1 of 100 gals.	5.30 a.m.	Boil water, make tea 1 pint per man with 2 oz. sugar per man. Serve at 6 a.m. with 1 oz. milk.
Sugar	2 oz.			
Milk	1 „			
		2 of 25 gals. (mealie meal uji)	6 a.m.	Put 6 oz. mealie meal flour per man in boiling water and $\frac{1}{2}$ lb. salt per 25 gallon pot and boil till 8 a.m. forming a thin gruel.
		5 dixies rice (5 gals.)	6 a.m.	Put 2 oz. rice per man in cold water with 1 oz. salt per 5 gallon dixie and boil till 7.30 a.m. Then evaporate off water over a slow fire.



DIET II (*cont.*).

Diet per man		Cooking pots per 500 men	Hour	Cooking instructions
Mealie uji	6 oz. }		8 a.m.	Serve mealie meal uji with rice.
Rice	2 .. }	2 of 25 gals. 1 of 25 gals.	8.30 a.m.	Put 6 oz. beans per man into cold water and boil hard till 11.30 a.m. Pour off water and serve at 12 noon.
			10 a.m.	Put rice 12 oz. per man into boiling water in dixies, boil and stir well till 11.30 a.m. Add salt 1 oz. per dixie and $\frac{1}{2}$ oz. ghee per man at 10.30 a.m.
			11.30 a.m.	Put rice over a small fire to evaporate off water.
Rice	12 oz. }		12 noon	Serve rice and beans.
Ghee	$\frac{1}{2}$ .. }			
Beans	6 .. }	2 of 25 gals. 1 of 25 gals. (meat) 1 of 25 gals. (bones)	2 p.m.	Put meat $\frac{1}{2}$ lb. per man with 1 lb. salt per 25 gallon pot into boiling water. Put bones from this meat into cold water and boil. Boil meat and bones till 5 p.m. Mix soup from each and give 1 pint per man.
			3 p.m.	Put 6 oz. mealie meal flour per man in boiling water and $\frac{1}{2}$ lb. per 25 gallon pot and boil till 5 p.m. forming a thin gruel.
Mealie meal uji	6 oz. }		5 p.m.	Serve mealie meal uji and meat and soup.
Meat	$\frac{1}{2}$ lb. }			
Soup	1 pint }			

The patient was treated with a fluid diet until his stools had been solid for two days and without any suspicion of blood or mucus. He was then put on to the semi-solid diet for two days, and if no diarrhoea showed itself in this time, he went on to full diet.

## GENERAL TREATMENT.

All dysentery cases were kept in bed as far as possible during the acute stage of the disease. Their mouths were cleaned twice daily with potassium permanganate lotion, and, if necessary, also in the intervals between these routine washings. They were also given a pint of water six times daily in addition to the fluid matter of the diet.

Most of these methods of treatment of bacillary dysentery by means of drugs were tried before vaccine treatment was commenced, and although a certain amount of improvement was attained in the reduction of the death and invaliding rate, yet as will be shown later from the results of treatment in 138 cases without anti-dysenteric vaccine, the results were far from encouraging.

## TREATMENT WITH ANTI-DYSENTERIC VACCINE.

The vaccine as used in this series of cases consisted of:

- (1) *B. Shiga* (3 strains) 500 millions.
- (2) *B. Flexner* (2 strains) 250 millions.
- (3) *B. Morgan* (3 strains) 750 millions.

Each strain was inoculated separately into a flask containing peptone bouillon, incubated for 72 hours, and then killed by the addition of 0.5 per

cent. carbolic acid. Each flask was then allowed to stand for 48 hours, and at the end of this time was tested for sterility. If sterile, the dead cultures were standardized, mixed in the above proportion, and bottled.

In order to compare the results of vaccine treatment, it was found necessary to record the cases under different groups, each group of cases being treated with a different dose of vaccine. As some of these groups contained only a very small number of cases, too inadequate to form any conclusions on, the groups were re-collected into two main divisions, namely *Division X* (Tables III–VI) into which all the early cases fell, and in which the dosage in the light of later experience was small, and *Division Y* (Tables VII–XIII), into which all the later cases fell, and in which the dosage was much larger.

It was further found that cases had to be divided into two main classes, namely “Effectives,” and “Invalids,” to admit of adequate comparison, “Effectives” being porters who were actually doing duty at the time of the onset of dysentery, or who had been passed as recruits for work: “Invalids” being porters who had been returned from posts nearer the actual fighting line, for disease contracted in the field which rendered them unfit for further duty. Thus under each group will be found two main classes, namely Effectives and Invalids.

Again it was obvious that it was unfair to take each case of dysentery as the same in severity, for some men reported sick as soon as the disease showed itself, others not for ten days afterwards, when toxaemia had set in and the patient had been reduced to a very weak state. The disease itself was also not always equal in severity. Consequently it was decided that some sort of subdivision of cases according to the severity of the disease and to the condition of the patient was essential.

The severity of the attack was adjudged on four main points, namely the symptoms, the amount of toxaemia, the amount of blood and mucus in the stools, and the number of evacuations daily.

The following classification was adopted:

(a) *Slight cases* in which the patient's physical condition was good, and the disease was mild and unaccompanied by toxaemia.

(b) *Medium cases* in which the patient's physical condition was good, but the disease was more severe, and in which a certain degree of toxaemia was seen.

(c) *Bad cases* in which the patient's physical condition was fair, but in which the disease was severe, and toxaemia marked.

(d) *Very bad cases* in which the patient's physical condition was poor, and in which the disease was severe and marked toxaemia was present.

(e) *Hopeless cases* in which the patient was admitted either in a comatose condition, or in which the patient was so emaciated and toxaemia so marked that no hope of the patient's recovery could be entertained.

It must here be remarked that the standard of physical condition was of necessity made much lower for invalids than for effectives, and that even then



possibly a considerably larger proportion of them should have been included amongst the "hopeless" class, but in every case the most optimistic view possible was taken.

A classification based on the characters of the case and the condition of the patient necessarily depends largely on the judgment of the observer, consequently in each group, a total is made of all cases included in that group, so that conclusions based on these totals are absolutely independent of any personal bias. It is unfortunate that whereas the vaccine cases were consecutive admissions to hospital, no case being excluded, the only non-vaccine cases available for comparison of which a record exists, were more or less selected men, in that all were "Effectives" and "Hopeless" cases were definitely excluded, this series having been recorded before vaccine treatment was started or even thought of. Consequently in order to compare total cases, it has been found essential to show in Table XV not only totals showing all cases, but also totals including all cases but "hopeless" ones.

As a further note it may be stated that even a comatose "hopeless" case sometimes recovered consciousness, and with the aid of stimulants and intravenous salines lived sometimes for a considerable number of days after admission, but in not a single case was the classification of "hopeless," which was always made on the day of admission, found unjustified, for no case in this class ever survived to be cited as a recovery.

A further division of porters was also necessary for statistical purposes. A certain number of porters received a dose of 1 c.c. (1500 million) of anti-dysenteric vaccine, others received a dose of 4 c.c. of anti-dysenteric vaccine, in both cases as a prophylactic measure when recruited. A large proportion of the cases received no prophylactic inoculation at all.

We have therefore the following outline classification under which all cases are brought.

(i) *Division.* X or Y, i.e. small or large therapeutic doses vaccine administered.

(ii) *Group.* I—X depending on therapeutic dose of vaccine administered.

(iii) *Class.* I. Effectives, II. Invalids.

(iv) *Sub-Class.*

A. Receiving no prophylactic inoculation.

B. Receiving 1 c.c. A.D. vaccine as a prophylactic.

C. Receiving 4 c.c. A.D. vaccine as a prophylactic.

(v) *Character of Case.* As described above.

Table III.

Group A. Not treated with anti-dysenteric vaccine.

Class	Character of case	Recoveries										Average number of days in hospital		
		Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases	
<i>Effectives</i> Prophylactic inoculation Nil	Slight	34	32	94.12	10	29.41	22	64.71	2	5.88	11.40	19.18	14.50	16.62
	Medium	43	34	79.07	6	13.95	28	65.12	9	20.93	10.00	20.68	18.33	18.69
	Bad	46	24	52.17	1	2.17	23	50.00	22	47.83	9.00	16.61	15.41	15.87
	Very bad	15	4	26.67	—	—	4	26.67	11	73.33	—	21.25	16.64	17.87
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—
	Total ...	138	94	68.12	17	12.32	77	55.80	44	31.88	10.76	19.06	16.27	17.15

Table IV.

Group B I. Initial dose. Variable, 5 million to 200 million.

Class	Character of case	Recoveries										Average number of days in hospital		
		Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases	
<i>Effectives</i> Prophylactic inoculation Nil	Slight	—	7	100.00	4	57.14	3	42.86	—	—	—	—	—	—
	Medium	7	5	83.33	3	50.00	2	33.33	1	16.67	16.25	17.66	5.00	16.86
	Bad	6	1	33.33	1	33.33	—	—	2	66.67	12.00	24.00	11.00	14.83
	Very bad	3	1	33.33	—	—	—	—	1	100.00	11.00	—	6.00	11.00
	Hopeless	1	—	—	—	—	—	—	—	—	—	—	—	6.00
	Total ...	17	13	76.47	8	47.06	5	29.41	4	23.53	14.00	20.20	8.25	14.47
<i>Invalids</i> Prophylactic inoculation Nil	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	1	—	—	—	—	—	100.00	1	100.00	—	—	7.00	7.00
	Very bad	—	—	—	—	—	—	—	—	—	—	—	—	—
	Hopeless	3	—	—	—	—	—	100.00	3	100.00	—	—	14.33	14.33
	Total ...	4	—	—	—	—	—	100.00	4	100.00	—	—	12.50	12.50



Table V.

GROUP B II. Initial dose. 90 million. (Doses every second day increased by half as much again.)

Class	Character of case	Recoveries										Average number of days in hospital			
		Total cases	Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases	
			No.	%	No.	%	No.	%	No.	%					
<i>Effectives</i> Prophylactic inoculation Nil	Slight	5	5	100-00	4	80-00	1	20-00	—	—	8-00	9-00	—	8-20	
	Medium	3	3	100-00	2	66-67	1	33-33	—	—	8-50	13-00	—	10-00	
	Bad	10	9	90-00	6	60-00	3	30-00	1	10-00	19-50	26-33	19-00	21-50	
	Very bad	8	5	62-50	3	37-50	2	25-00	3	37-50	19-67	39-50	27-00	27-37	
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Total ...	26	22	84-61	15	57-69	7	26-92	4	15-39	15-00	25-71	25-00	19-42	
<i>Invalids</i> Prophylactic inoculation Nil	Slight	9	8	88-89	2	22-22	6	66-67	1	11-11	12-50	14-50	1-00	12-55	
	Medium	2	1	50-00	1	50-00	—	—	1	50-00	10-00	—	18-00	14-00	
	Bad	8	4	50-00	2	25-00	2	25-00	4	50-00	34-00	8-50	24-75	23-00	
	Very bad	10	4	40-00	—	—	4	40-00	6	60-00	—	78-25	19-83	43-20	
	Hopeless	3	—	—	—	—	—	—	3	100-00	—	—	24-00	24-00	
	Total ...	32	17	53-12	5	15-62	12	37-50	15	46-88	20-60	34-75	20-60	25-91	

Table VI.

GROUP B III. Initial dose. 90 million. (Doses repeated weekly increased by half as much again.)

Class	Character of case	Total cases	Recoveries						Average number of days in hospital					
			Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases
			No.	%	No.	%	No.	%	No.	%				
<i>Effectives</i> Prophylactic inoculation Nil	Slight	2	2	100-00	2	100-00	—	—	—	—	9-00	—	—	9-00
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	2	—	—	—	—	—	—	2	100-00	—	—	52-50	52-50
	Hopeless	1	—	—	—	—	—	—	1	100-00	—	—	29-00	29-00
	Total ...	5	2	40-00	2	40-00	—	—	3	60-00	9-00	—	44-66	30-40
<i>Invalids</i> Prophylactic inoculation Nil	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	1	1	100-00	—	—	1	100-00	—	—	—	27-00	—	27-00
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	—	—	—	—	—	—	—	—	—	—	—	—	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—
	Total ...	1	1	100-00	—	—	1	100-00	—	—	—	27-00	—	27-00

Table VII.

Group B IV. Initial dose. 180 million. (Doses repeated weekly increased by half as much again.)

Class		Character of case	Recoveries										Average number of days in hospital			
			Total cases	Total		To duty		Invalided		Deaths		Duty				
				No.	%	No.	%	No.	%	No.	%		No.	%		
<i>Effectives</i> Prophylactic inoculation Nil	Slight	5	5	100.00	4	80.00	1	20.00	—	—	—	16.00	10.00	—	14.80	
	Medium	5	5	100.00	4	80.00	1	20.00	—	—	—	9.25	11.00	—	9.60	
	Bad	4	4	100.00	3	75.00	1	25.00	—	—	—	14.67	35.00	—	19.75	
	Very bad	4	2	50.00	2	50.00	—	—	—	2	50.00	49.00	—	25.50	37.25	
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Total ...		18	16	88.89	13	72.22	3	16.67	3	16.67	2	11.11	18.69	18.67	25.50	19.44
<i>Invalids</i> Prophylactic inoculation Nil	Slight	1	1	100.00	1	100.00	—	—	—	—	—	8.00	—	—	8.00	
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Very bad	2	2	100.00	—	—	2	100.00	—	—	—	—	29.00	—	29.00	
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Total ...		3	3	100.00	1	33.33	2	66.67	2	66.67	—	—	8.00	29.00	—	22.00

Table VIII.

Group B V. Initial dose. 750 million. (Doses doubled at weekly intervals.)

Class		Character of case	Recoveries										Average number of days in hospital			
			Total cases	Total		To duty		Invalided		Deaths		Duty				
				No.	%	No.	%	No.	%	No.	%		No.	%		
<i>Effectives</i> Prophylactic inoculation Nil	Slight	4	4	100.00	4	100.00	—	—	—	—	—	7.50	—	—	7.50	
	Medium	3	3	100.00	3	100.00	—	—	—	—	—	12.33	—	—	12.33	
	Bad	4	4	100.00	4	100.00	—	—	—	—	—	11.25	—	—	11.25	
	Very bad	2	1	50.00	1	50.00	—	—	—	1	50.00	52.00	—	33.00	42.50	
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Total ...		13	12	92.30	12	92.30	—	—	—	1	7.70	13.66	—	33.00	15.15	
<i>Effectives</i> Prophylactic inoculation 1 c.c.	Slight	1	1	100.00	1	100.00	—	—	—	—	—	13.00	—	—	13.00	
	Medium	1	—	—	—	—	—	—	1	100.00	—	—	—	18.00	18.00	
	Bad	2	2	100.00	2	100.00	—	—	—	—	—	14.50	—	—	14.50	
	Very bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Total ...		4	3	75.00	3	75.00	—	—	—	1	25.00	14.00	—	18.00	15.00	
<i>Invalids</i> Prophylactic inoculation Nil	Slight	3	1	33.33	—	—	1	33.33	2	66.67	—	—	9.00	9.50	9.33	
	Medium	1	1	100.00	1	100.00	—	—	—	—	—	33.00	—	—	33.00	
	Bad	7	6	85.71	5	71.43	1	14.28	1	14.28	1	43.20	65.00	10.00	41.57	
	Very bad	1	—	—	—	—	—	—	1	100.00	—	—	—	12.00	12.00	
	Hopeless	2	—	—	—	—	—	—	2	100.00	—	—	—	49.00	49.00	
Total ...		14	8	57.14	6	42.86	2	14.28	6	42.86	6	41.50	37.00	23.17	33.00	



Table IX.  
GROUP B VI. Initial dose. 1500 million. (Doses doubled at weekly intervals.)

Class	Character of case	Recoveries										Average number of days in hospital			
		Total cases	Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases	
			No.	%	No.	%	No.	%	No.	%					
<i>Effectives</i> Prophylactic inoculation Nil	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	7	7	100·00	7	100·00	—	—	—	—	16·71	—	—	16·71	—
	Bad	2	2	100·00	2	100·00	—	—	—	—	11·00	—	—	11·00	—
	Very bad	3	3	100·00	3	100·00	—	—	—	—	24·67	—	—	24·67	—
	Hopeless	3	—	—	—	—	—	—	3	100·00	—	—	60·33	60·33	—
Total ...		15	12	80·00	12	80·00	—	—	3	20·00	17·75	—	60·33	60·33	26·26
<i>Effectives</i> Prophylactic inoculation I c.c.	Slight	1	1	100·00	1	100·00	—	—	—	—	15·00	—	—	15·00	—
	Medium	13	13	100·00	13	100·00	—	—	—	—	12·46	—	—	12·46	—
	Bad	6	5	83·33	5	83·33	—	—	1	16·67	25·60	—	9·00	22·83	—
	Very bad	7	5	71·43	5	71·43	—	—	2	28·57	41·40	—	31·50	38·56	—
	Hopeless	3	—	—	—	—	—	—	3	100·00	—	—	12·00	12·00	—
Total ...		30	24	80·00	24	80·00	—	—	6	20·00	21·33	—	36·00	20·67	—
<i>Invalids</i> Prophylactic inoculation Nil	Slight	2	2	100·00	—	—	2	100·00	—	—	—	9·50	—	9·50	—
	Medium	14	14	100·00	9	64·29	5	35·71	—	—	16·44	21·40	—	18·21	—
	Bad	11	9	81·82	6	54·54	3	27·28	2	18·18	38·67	31·33	51·00	38·91	—
	Very bad	12	4	33·33	3	25·00	1	8·33	8	66·67	31·33	13·00	28·75	28·08	—
	Hopeless	6	—	—	—	—	—	—	6	100·00	—	—	21·83	21·83	—
Total ...		45	29	64·44	18	40·00	11	24·44	16	35·56	26·33	21·18	28·94	26·00	—

Table X. GROUP B VII. Initial dose. 3000 million. (Doses doubled at weekly intervals.)

Class	Character of case	Recoveries										Average number of days in hospital			
		Total		To duty		Invalided		Deaths		Duty		Invalided		Died	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<i>Effectives</i> Prophylactic inoculation Nil	Slight	2	100.00	2	100.00	—	—	—	—	—	—	—	—	—	8.50
	Medium	8	100.00	8	100.00	—	—	—	—	—	—	—	—	—	19.00
	Bad	12	91.67	11	91.67	—	—	1	8.33	—	—	—	—	53.00	21.00
	Very bad	17	76.47	12	70.59	1	5.88	4	23.53	25.42	—	48.00	—	27.50	27.24
	Hopeless	3	—	—	—	—	—	3	100.00	—	—	—	—	16.66	16.66
Total ...		42	34	80.95	33	78.75	1	2.38	8	19.05	20.39	48.00	26.62	22.24	—
<i>Effectives</i> Prophylactic inoculation 1 e.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	2	100.00	2	100.00	—	—	—	—	—	—	—	—	—	18.50
	Bad	5	100.00	5	100.00	—	—	—	—	—	—	—	—	—	16.80
	Very bad	8	62.50	4	50.00	1	12.50	3	37.50	21.00	—	53.00	—	50.33	36.00
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		15	12	80.00	11	73.33	1	6.67	3	20.00	18.64	53.00	50.33	27.27	27.27
<i>Effectives</i> Prophylactic inoculation 4 e.c.	Slight	3	100.00	3	100.00	—	—	—	—	—	—	—	—	—	10.67
	Medium	24	87.50	21	87.50	—	—	3	12.50	17.67	—	—	—	39.00	20.33
	Bad	51	86.27	42	82.35	2	3.92	7	13.73	22.62	—	86.00	—	20.57	24.82
	Very bad	53	69.81	35	66.04	2	3.77	16	30.19	29.00	—	98.00	—	33.19	30.98
	Hopeless	7	—	—	—	—	—	7	100.00	—	—	—	—	17.86	17.86
Total ...		138	105	76.09	101	73.19	4	2.90	33	23.91	23.45	92.00	27.79	25.75	25.75
<i>Invalids</i> Prophylactic inoculation Nil	Slight	2	100.00	2	100.00	—	—	—	—	—	—	—	—	—	7.50
	Medium	3	100.00	3	100.00	—	—	—	—	—	—	—	—	—	13.67
	Bad	3	66.67	2	66.67	—	—	1	33.33	22.50	—	—	—	8.00	17.67
	Very bad	6	33.33	2	33.33	—	—	4	66.67	36.00	—	—	—	27.25	30.17
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		14	9	64.29	9	64.29	—	—	5	35.71	19.22	—	23.40	20.71	—
<i>Invalids</i> Prophylactic inoculation 1 e.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	4	50.00	1	25.00	1	25.00	2	50.00	30.00	—	75.00	—	15.50	34.00
	Bad	2	50.00	1	50.00	—	—	1	50.00	13.00	—	—	—	9.00	11.00
	Very bad	14	3	21.43	2	14.29	1	7.14	11	78.57	20.50	73.00	—	10.64	24.36
	Hopeless	3	—	—	—	—	—	3	100.00	—	—	—	—	7.33	7.33
Total ...		23	6	26.09	4	17.39	2	8.70	17	73.91	21.00	74.00	17.00	22.65	22.65
<i>Invalids</i> Prophylactic inoculation 4 e.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	100.00	3	100.00	—	—	—	—	35.33	—	—	—	—	35.33
	Bad	10	50.00	3	30.00	2	20.00	5	50.00	16.67	—	52.50	—	24.60	27.80
	Very bad	11	3	27.27	3	27.27	—	—	8	72.73	49.00	—	—	22.63	29.82
	Hopeless	9	—	—	—	—	—	9	100.00	—	—	—	—	9.00	9.00
Total ...		33	11	33.33	9	27.27	2	6.06	22	66.67	33.67	52.50	17.50	24.03	24.03



Table XI.

Group B VIII. Initial dose. 6000 million. (Doses doubled at weekly intervals.)

Class	Character of case	Recoveries										Average number of days in hospital		
		Total cases	Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases
			No.	%	No.	%	No.	%	No.	%				
<i>Effectives</i> Prophylactic inoculation Nil	Slight	1	1	100.00	1	100.00	—	—	—	—	11.00	—	—	11.00
	Medium	3	3	100.00	3	100.00	—	—	—	—	24.33	—	—	24.33
	Bad	8	7	87.50	7	87.50	—	—	1	12.50	24.57	—	43.00	26.88
	Very bad	9	4	44.44	4	44.44	—	—	5	55.56	38.50	—	36.80	37.55
	Hopeless	1	—	—	—	—	—	—	1	100.00	—	—	35.00	35.00
Total ...		22	15	68.18	15	68.18	—	—	7	31.82	27.33	—	37.43	30.55
<i>Effectives</i> Prophylactic inoculation I e.c.	Slight	1	1	100.00	1	100.00	—	—	—	—	10.00	—	—	10.00
	Medium	11	11	100.00	11	100.00	—	—	—	—	20.36	—	—	20.36
	Bad	8	7	87.50	7	87.50	—	—	1	12.50	22.14	—	30.00	23.13
	Very bad	12	9	75.00	7	58.33	2	16.67	3	25.00	23.71	68.50	28.67	32.42
	Hopeless	2	—	—	—	—	—	—	2	100.00	—	—	47.50	47.50
Total ...		34	28	82.35	26	76.47	2	5.88	6	17.65	21.35	68.50	35.17	26.56
<i>Effectives</i> Prophylactic inoculation 4 e.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	2	1	50.00	1	50.00	—	—	1	50.00	19.00	—	75.00	47.00
	Hopeless	1	—	—	—	—	—	—	1	100.00	—	—	50.00	50.00
Total ...		3	1	33.33	1	33.33	—	—	2	66.67	19.00	—	62.50	48.00
<i>Invalids</i> Prophylactic inoculation Nil	Slight	2	2	100.00	2	100.00	—	—	—	—	17.00	—	—	17.00
	Medium	3	3	100.00	1	33.33	2	66.67	—	—	19.00	21.50	—	20.67
	Bad	6	2	33.33	2	33.33	—	—	4	66.67	13.50	—	76.75	55.67
	Very bad	15	3	20.00	1	6.67	2	13.33	12	80.00	14.00	32.00	32.17	30.93
	Hopeless	9	—	—	—	—	—	—	9	100.00	—	—	7.78	7.78
Total ...		35	10	28.57	6	17.14	4	11.43	25	71.43	15.67	26.75	30.52	27.54
<i>Invalids</i> Prophylactic inoculation I e.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	3	100.00	2	66.67	1	33.33	—	—	18.00	28.00	—	21.33
	Bad	10	6	60.00	5	50.00	1	10.00	4	40.00	32.60	78.00	63.25	49.40
	Very bad	16	6	37.50	6	37.50	—	—	10	62.50	53.87	—	19.20	32.19
	Hopeless	7	—	—	—	—	—	—	7	100.00	—	—	11.14	11.14
Total ...		36	15	41.67	13	36.11	2	5.56	21	58.33	40.15	53.00	24.90	31.97

Table XII.

Group B ix. Initial dose. 1500 million. (Dose doubled every three days.)

Class	Character of case	Recoveries										Average number of days in hospital			
		Total		To duty		Invalided		Deaths		Duty		Invalided	Died	All cases	
		No.	%	No.	%	No.	%	No.	%	No.	%				
<i>Effectives</i> Prophylactic inoculation Nil	Slight	7	100·00	7	100·00	—	—	—	—	18·14	—	—	—	18·14	—
	Medium	31	100·00	30	96·77	1	3·23	—	—	14·73	—	31·00	—	15·26	—
	Bad	67	91·04	52	77·61	9	13·43	6	8·96	22·62	—	37·56	21·50	24·52	—
	Very bad	24	58·33	6	25·00	8	33·33	10	41·67	26·33	—	30·37	14·80	22·87	—
	Hopeless	2	—	—	—	—	—	2	100·00	—	—	—	6·50	6·50	—
Total ...		131	86·26	95	72·52	18	13·74	18	13·74	20·03	—	34·00	16·11	21·41	—
<i>Effectives</i> Prophylactic inoculation 4 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	100·00	2	66·67	1	33·33	—	—	21·50	—	44·00	—	29·00	—
	Bad	5	100·00	5	100·00	—	—	—	—	23·80	—	—	—	23·80	—
	Very bad	1	100·00	1	100·00	—	—	—	—	41·00	—	—	—	41·00	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		9	9	8	88·89	1	11·11	—	—	25·37	—	44·00	—	27·44	—
<i>Invalids</i> Prophylactic inoculation Nil	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	100·00	—	—	3	100·00	—	—	—	—	23·00	—	23·00	—
	Bad	10	90·00	1	10·00	8	80·00	1	10·00	24·00	—	36·87	47·00	29·20	—
	Very bad	11	8	—	—	8	72·73	3	27·27	—	—	25·50	16·00	22·91	—
	Hopeless	2	—	—	—	—	—	2	100·00	—	—	—	11·00	11·00	—
Total ...		26	20	1	3·85	19	73·07	6	23·08	24·00	—	26·00	19·50	24·42	—
<i>Invalids</i> Prophylactic inoculation 1 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	1	100·00	—	—	1	100·00	—	—	—	—	15·00	—	15·00	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		1	1	—	—	1	100·00	—	—	—	—	15·00	—	15·00	—
<i>Invalids</i> Prophylactic inoculation 4 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	7	100·00	—	—	7	100·00	—	—	—	—	24·57	—	24·57	—
	Very bad	9	4	—	—	4	44·44	5	55·56	—	—	38·75	14·20	25·11	—
	Hopeless	2	—	—	—	—	—	2	100·00	—	—	—	7·50	7·50	—
Total ...		18	11	—	—	11	61·11	7	38·89	—	—	29·73	12·29	22·94	—



Table XIII.

GROUP B X. Initial dose. 12,000 million. (Repeated weekly.)

Class	Character of case	Recoveries										Average number of days in hospital				
		Total cases	Total		To duty		Invalided		Deaths		Duty					Invalided
			No.	%	No.	%	No.	%	No.	%		No.	%			
<i>Effectives</i> Prophylactic inoculation 1 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	1	—	—	—	—	—	—	—	1	100.00	—	—	23.00	—	23.00
	Very bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Total ...	1	—	—	—	—	—	—	—	1	100.00	—	—	23.00	—	23.00

Table XIV.

DIVISION Y. Summary of Tables VII to XIII.

Class	Character of case	Recoveries										Average number of days in hospital			
		Total cases	Total		To duty		Invalided		Deaths		Duty				Invalided
			No.	%	No.	%	No.	%	No.	%		No.	%		
<i>Effectives</i> Prophylactic inoculation Nil	Slight	19	100.00	18	94.74	1	5.26	—	—	—	—	13.83	10.00	—	13.63
	Medium	57	100.00	55	96.49	2	3.51	—	—	—	—	13.78	21.00	—	14.03
	Bad	97	91.75	79	81.44	10	10.31	8	8.25	—	—	20.99	37.30	28.13	23.26
	Very bad	59	62.71	28	47.46	9	15.25	22	37.29	37	100.00	30.03	32.33	23.91	28.10
	Hopeless	9	—	—	—	—	—	9	100.00	—	—	—	—	31.00	31.00
	Total ...	241	202	83.82	180	74.69	22	9.13	39	16.18	—	19.44	32.55	26.41	21.79
<i>Effectives</i> Prophylactic inoculation 1 c.c.	Slight	3	100.00	3	100.00	—	—	—	—	—	—	12.67	—	—	12.67
	Medium	27	96.29	26	96.29	—	—	1	3.71	—	—	16.27	—	18.00	16.33
	Bad	22	86.36	19	86.36	—	—	3	13.64	—	—	20.84	—	20.67	20.82
	Very bad	27	70.37	16	59.26	3	11.11	8	29.63	—	—	28.56	63.33	37.50	35.07
	Hopeless	5	—	—	—	—	—	5	100.00	—	—	—	—	26.20	26.20
	Total ...	84	67	79.76	64	76.19	3	3.57	17	20.24	—	20.53	63.33	30.06	23.99

Table XIV—*contd.*  
DIVISION Y. Summary of Tables VII to XIII.—*contd.*

Class	Character of case	Recoveries										Average number of days in hospital			
		Total cases		Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases
		No.	%	No.	%	No.	%	No.	%	No.	%				
<i>Effectives</i> Prophylactic inoculation 4 c.c.	Slight	3	100.00	3	100.00	—	—	—	—	—	—	10.67	—	—	10.67
	Medium	27	88.89	23	85.19	1	3.70	1	3.70	3	11.11	18.00	44.00	39.00	21.29
	Bad	56	87.50	47	83.93	2	3.57	2	3.57	7	12.50	22.74	86.00	20.57	24.73
	Very bad	56	69.64	37	66.07	2	3.57	2	3.57	17	30.36	29.05	83.00	35.65	33.52
	Hopeless	8	—	—	—	—	—	—	—	8	100.00	—	—	21.87	21.87
Total ...		150	76.67	110	73.33	5	3.34	5	3.34	35	23.33	23.55	82.40	29.77	26.96
<i>Invalids</i> Prophylactic inoculation Nil	Slight	10	80.00	5	50.00	3	30.00	3	30.00	2	20.00	11.40	9.33	9.50	10.40
	Medium	24	100.00	14	58.33	10	41.67	10	41.67	—	—	17.21	21.90	—	19.17
	Bad	37	75.68	16	43.24	12	32.44	12	32.44	9	24.32	34.00	37.83	52.67	39.78
	Very bad	47	40.43	6	12.77	13	27.66	13	27.66	28	59.57	30.00	26.08	28.04	27.74
	Hopeless	19	—	—	—	—	—	—	—	19	100.00	—	—	16.89	16.89
Total ...		137	57.66	41	29.93	38	27.73	38	27.73	58	42.34	24.93	27.37	27.57	26.72
<i>Invalids</i> Prophylactic inoculation 1 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	7	71.43	3	42.86	2	28.57	2	28.57	2	28.57	22.00	51.50	15.50	28.57
	Bad	12	58.33	6	50.00	1	8.33	1	8.33	5	41.67	29.33	78.00	52.40	43.00
	Very bad	31	32.26	8	25.81	2	6.45	2	6.45	21	67.74	45.50	44.00	14.71	24.55
	Hopeless	10	—	—	—	—	—	—	—	10	100.00	—	—	10.00	10.00
Total ...		60	36.67	17	28.33	5	8.34	5	8.34	38	63.33	35.65	53.80	18.47	26.28
<i>Invalids</i> Prophylactic inoculation 4 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	100.00	3	100.00	—	—	—	—	—	—	35.33	—	—	35.33
	Bad	17	70.59	3	17.65	9	52.94	9	52.94	5	29.41	16.67	30.78	24.60	26.47
	Very bad	20	35.00	3	15.00	4	20.00	4	20.00	13	65.00	49.00	38.75	19.38	27.70
	Hopeless	11	—	—	—	—	—	—	—	11	100.00	—	—	87.28	87.28
Total ...		51	43.14	9	17.65	13	25.49	13	25.49	29	56.86	33.67	33.23	16.24	23.65



Table XV.

Summary of all Vaccine cases in Divisions X and Y.

Class	Character of case	Recoveries										Average number of days in hospital							
		Total		To duty		Invalided		Deaths		Duty						Invalided		All cases	
		No.	%	No.	%	No.	%	No.	%	No.	%					No.	%	No.	%
Division X <i>Effectives</i>	Slight	7	100-00	6	85-71	1	14-29	—	—	—	—	8-33	9-00	—	8-43				
	Medium	10	100-00	6	60-00	4	40-00	—	—	—	—	13-67	16-50	—	14-80				
	Bad	16	14	87-50	9	56-25	5	30-25	2	12-50	—	17-00	25-40	12-00	19-00				
	Very bad	13	6	46-15	4	30-77	2	15-38	7	53-85	—	17-50	39-50	29-71	27-46				
	Hopeless	2	—	—	—	—	—	—	2	100-00	—	—	—	17-50	17-50				
Total all cases		48	37	77-08	25	52-08	12	25-00	11	22-92	—	14-20	23-42	24-27	18-77				
Total excluding "Hopeless" cases		46	37	80-43	25	54-35	12	26-08	9	19-57	—	14-20	23-42	25-78	18-83				
Division X <i>Invalids</i>	Slight	9	8	88-89	2	22-22	6	66-67	1	11-11	—	12-50	14-50	1-00	12-55				
	Medium	3	2	66-67	1	33-33	1	33-33	1	33-33	—	10-00	27-00	18-00	18-33				
	Bad	9	4	44-44	2	22-22	2	22-22	5	55-56	—	34-00	8-50	21-20	21-22				
	Very bad	10	4	40-00	—	—	4	40-00	6	60-00	—	—	78-25	19-83	43-20				
	Hopeless	6	—	—	—	—	—	—	6	100-00	—	—	—	19-17	19-17				
Total all cases		37	18	48-65	5	13-51	13	35-14	19	51-35	—	20-60	34-15	18-89	24-49				
Total excluding "Hopeless" cases		31	18	58-06	5	16-13	13	41-93	13	41-93	—	20-60	34-15	18-77	25-52				
Division Y <i>Effectives</i>	Slight	25	25	100-00	24	96-00	1	4-00	—	—	—	13-29	10-00	—	13-16				
	Medium	111	107	96-39	104	93-69	3	2-70	4	3-61	—	15-34	28-67	33-75	16-36				
	Bad	175	157	89-71	145	82-80	12	6-91	18	10-29	—	21-54	45-42	23-94	23-48				
	Very bad	142	95	66-90	81	57-04	14	8-86	47	33-10	—	29-29	48-36	30-47	31-56				
	Hopeless	22	—	—	—	—	—	—	22	100-00	—	—	—	26-59	26-59				
Total all cases		475	384	80-84	354	74-53	30	6-31	91	19-16	—	20-93	43-93	28-38	23-81				
Total excluding "Hopeless" cases		453	384	84-77	354	78-15	30	6-62	69	15-23	—	20-93	43-93	28-96	23-68				
Division Y <i>Invalids</i>	Slight	10	8	80-00	5	50-00	3	30-00	2	20-00	—	11-40	9-33	9-50	10-40				
	Medium	34	32	94-12	20	58-82	12	35-30	2	5-88	—	20-65	26-83	15-50	22-53				
	Bad	66	47	71-21	25	37-88	22	33-33	19	28-79	—	30-80	36-77	45-21	36-94				
	Very bad	98	36	36-73	17	17-35	19	19-38	62	63-27	—	40-65	30-63	21-71	26-72				
	Hopeless	40	—	—	—	—	—	—	40	100-00	—	—	—	12-93	12-93				
Total all cases		248	123	49-60	67	27-02	56	22-58	125	50-40	—	28-82	31-09	22-18	25-98				
Total excluding "Hopeless" cases		208	123	59-13	67	32-21	56	26-92	85	40-87	—	28-82	31-09	26-53	28-49				

Having therefore explained the method of classification used, we can turn to the other point, namely what conclusions can be drawn from the recorded results of these cases?

(1) In the first place we may note that, as far as the death rate is concerned, there is practically no difference in the results of vaccine treatment between cases which received prophylactic inoculation and those which did not, indeed if anything the cases which received a prophylactic inoculation did worse and had a higher death rate than those which received no such inoculation. The real difference between cases which received a prophylactic inoculation and those which did not, lies in the percentage of those returning to duty after the disease. Here the cases which received a prophylactic inoculation show a considerably higher proportion of men returning to duty than cases which received no prophylactic inoculation, among effective porters. The opposite is the case amongst invalids, but here a fallacy may possibly be introduced as it is still uncertain how long any immunity conferred by prophylactic inoculation lasts. It seems probable from statistics at present available, that the immunity, if any, only lasts for a comparatively short time, in which case invalids coming under the head of prophylactically inoculated, should really be classed among the non-inoculated.

(2) The only available records of cases not treated therapeutically with vaccine are of effective porters from which "hopeless" cases were excluded. On comparison of the death rate amongst these men, which was 31·38 per cent., with the cases treated with vaccine, we see that amongst effective porters treated with small doses of vaccine, the death rate was lowered to 22·92 per cent. whilst amongst a similar class of men treated with large doses of vaccine, it fell to 19·16 per cent. Here there is no room for any individual bias to enter, for all the vaccine cases were consecutive admissions to hospital whilst the control cases were to a large extent selected cases. If we exclude "hopeless" cases from the vaccine series, the death rate falls to 19·57 per cent. and 15·25 per cent. respectively. Therefore from these figures it may be concluded that the therapeutic use of the vaccine definitely influences the course of the disease for good and markedly reduces the death rate from bacillary dysentery when administered in sufficiently large doses.

In the case of invalids there are unfortunately no records of cases untreated with vaccine for comparison, and it is only possible from the recollection of the writer of the feeling of utter hopelessness in dealing with these cases in pre-vaccine days, to give an assurance that, poor as are the results shown for invalids under vaccine treatment, matters were far worse in the days when no vaccine was available. If the figures are thought to show an appallingly high death rate, it must be remembered that these invalids had been returned as useless to the forces in the field, that many of them were frightfully wasted from disease, and with no resistance left to protect them from the invasion of fresh hostile bacteria, and one may even look upon it as a triumph of medicine that, of their number, we were able to send back to the front even as small a



proportion as 13·51 per cent. in one set of cases and 27·02 per cent. in the other.

It will be noted that the statistics show improvement in the death rate with vaccine, not only amongst the milder cases, but also amongst the "very bad" or toxæmic class.

Besides comparison of the death rate, it is interesting to compare the statistics showing the percentage of cases returned to duty, a matter of great importance in a campaign when every effective porter was an asset to our forces. In pre-vaccine days it was considered that, if a man went into hospital suffering from dysentery, it was almost a certainty that, if he escaped death, he would be sent home as an invalid, only 21·32 per cent. being returned to duty. Compare with this the figures of effective porters treated with vaccine. Even with small doses, inadequate as they seemed in the light of later experience, over 50 per cent. of cases of dysentery were returned for further service at a time when the standard of fitness for duty was very high, and with the larger doses almost 75 per cent. of treated cases were sent back to duty. As has been mentioned above, a small proportion from 13·51 per cent. up to 27·03 per cent. of men returned as invalids and admitted to hospital with dysentery, were saved for a further period of usefulness in the field, a rather larger proportion than were returned to duty from the pre-vaccine series of selected effectives. From the point of view therefore of military efficiency, the vaccine was an important asset in the medical armamentarium.

In the statistics of treated cases, the average number of days spent in hospital has been included. Apparently the vaccine has very little effect on the number of days cases remained under treatment, though the average time in the hospital of vaccine cases was undoubtedly increased by the longer time cases, which eventually died, remained in hospital. In the pre-vaccine series of porters, similar cases died in a much shorter time.

(3) As regards the dosage of vaccine advisable, full statistics of each different dosage tried are included in the Tables. Certain of these were used with too small a number of cases to be useful for any statistical purpose, but on consideration of the various groups, when a sufficient number of cases are included to make conclusions reliable, it would seem that the results obtained with a dosage of 2 c.c. (3000 million), 4 c.c. and 8 c.c. vaccine on the first, fourth and eighth days were the most satisfactory and this is the dosage which is now recommended for general use.

(4) A small series of cases was tried with autogeneous dysentery vaccines, but the numbers were too few to admit of statistical use. The general impression gathered, however, was that there was no advantage in their use over the polyvalent dysentery vaccine.

From the above facts it is recommended that the treatment of dysentery cases should be carried out on the following lines:

(1) Eliminate amoebic, bilharzial, and malarial dysentery by microscopical examination.

(2) Put the patient to bed.

(3) Give an aperient, and keep the bowels open by the use of magnesium sulphate in small doses three or more times a day.

(4) Administer *on the first day* or at the first possible moment, an initial dose of 2 c.c. (3000 million) of the polyvalent anti-dysenteric vaccine, followed by 4 c.c. on the fourth day and 8 c.c. on the eighth day, if necessary.

(5) Keep the patient on a light diet till the stools are normal in appearance and semi-solid, then gradually change to normal diet, being guided by the appearance of the stools.

(6) In the event of intractable diarrhoea setting in, give *B. coli communis* vaccine in  $\frac{1}{2}$  c.c., 1 c.c., and 2 c.c. doses at intervals of 3 days subcutaneously and by the mouth Pulvis Rhei and sodii bicarbonas one drachm of each thrice daily till the stools become normal in colour and tenesmus disappears.

It may be mentioned that, as post dysenteric diarrhoea was so common, and prolonged treatment to such an extent, an attempt was made to combine the treatments with anti-dysenteric vaccine and *B. coli* vaccine by giving them simultaneously, but no advantage was gained, the *B. coli* failing to produce the same effect as it does when it follows anti-dysenteric vaccine.

It must be noted that certain cases clinically and microscopically of bacillary type failed to respond at all to the anti-dysenteric vaccine. An explanation of this class of case may possibly be that heterologous dysentery bacilli were the causal organisms, or again the case may have been due to *Entamoeba histolytica* originally and treated as such, but secondary organisms, such as *B. coli communis*, may have got implanted on the healing amoebic ulceration, and have kept up the dysenteric condition. Certainly some of these cases responded markedly to a *B. coli* vaccine.

It is also worth recording that the vaccine in certain patients produced an immediate effect, the stools diminishing rapidly in number, their character improving, and the general condition of the patient becoming much better. It has happened too often to be merely coincidence, and it must be concluded therefore that one of its effects may be the immediate stimulation of phagocytosis. Independent testimony to this and also to the value of the vaccine as a therapeutic agent has been received from other observers from whose reports the following statements are quoted.

Capt. Shircore, E.A.M.S., Native Civil Hospital, Mombasa: "In my experience the best results obtained with anti-dysenteric vaccine are in chronic dysenteries. Its effects are immediate, *i.e.* within 18 to 24 hours, and whatever may be the explanation of such rapid action, nevertheless this result can be observed clinically. I regard it as a valuable therapeutic agent and would use it without fail in the type of cases mentioned in preference to any other line of treatment that I know of; and with confidence that if it was going to do good at all, it would do so promptly."

Capt. Mackinnon, E.A.M.S., K.A.R. Hospital, M'bagathi: "Although the preparation is a vaccine, I have usually found that there is an immediate



marked improvement after the first dose in nearly all cases in which a cure is obtained. There is usually a marked reaction and the temperature may run up to 103° F. or even more. I have noticed in several cases that, on the day following the inoculation, patients who have been passing 15 to 20 motions a day are suddenly reduced to one, or even to none, and show no signs of relapse thereafter."

Other observers have also noted a similar result with anti-dysenteric vaccine. See Skalski and Sterling (1917, *Deutsche med. Wochenschr.* XLIII. 713, abstract in *Trop. Dis. Bull.* x. 140), and Margolis (1917, *Deutsche med. Wochenschr.* XLIII. 783, abstract in *Bull. Inst. Pasteur*, xv. 557).

A testimony to the efficacy of the vaccine in treatment was given to the writer by two of his hospital dressers, who contracted dysentery while on duty, and insisted on being treated with the vaccine, although as a rule a native is distinctly averse to having needles thrust into him.

As a fact which requires further investigation, the writer wishes to record a general constitutional effect of the anti-dysenteric vaccine. A patient who had been in hospital for some considerable time with a bad ulcer of the foot, and a chronic inflammatory condition of the calf of the leg probably resulting therefrom, contracted dysentery. He was treated with anti-dysenteric vaccine in the usual way, and not only rapidly recovered from the intestinal disease, but the ulcer and the inflamed leg also became cured although they had resisted all other forms of treatment for months. So marked was the effect, that a case with a similar condition of ulcer and chronic cellulitis of the leg was tentatively given a dose of anti-dysenteric vaccine, and immediately cleared up. Unfortunately no other cases with exactly similar lesions have occurred, and no smear was taken from the ulcers of these two patients. The results with the vaccine are suggestive, however, and it has yet to be investigated whether dysentery bacilli do or do not occur in similar ulcers of the skin.

In conclusion the writer's thanks are due to Dr P. H. Ross, Director of Laboratories, E.A.P., whose suggestion to sterilise the vaccine with carbolic acid instead of heat made it possible to use large doses in treatment, to Dr J. Harvey Pirie, Institute of Medical Research, Johannesburg, from whose work on the dysentery bacilli of E. Africa, the idea of vaccine treatment was first formulated by the writer, and to both these officials for the way in which, under great difficulties and stress of other work, they so maintained the supply of the vaccine as to meet even excessive calls on it. The writer has also to thank Capt. J. A. M. Clarke, R.A.M.C., who supplied the records of cases detailed in Group B IX, and to Colonel Clemesha, D.D.M.S., E.A.E.F., for permission to publish this paper.

## CATS AND HUMAN DIPHTHERIA.

By WILLIAM G. SAVAGE, M.D.,  
*County Medical Officer of Health, Somerset.*

It is a widely accepted belief amongst medical men, particularly those who are Medical Officers of Health, that cats may suffer from diphtheria and convey it to human cases and that they are a not uncommon source of infection. It is of considerable practical importance to determine to what extent this belief is based upon reliable scientific data or whether it is another of those opinions, still far too prevalent, which arise from the acceptance of insufficiently tested and incorrectly interpreted observations.

In dealing with this subject I propose to give first a brief account of the available evidence upon which the association of cats with diphtheria has been built, then a summary of my own investigations and lastly a critical consideration of the whole of the data available.

### EVIDENCE ASSOCIATING CATS WITH HUMAN DIPHTHERIA.

This is both experimental and epidemiological.

Of the experimental work the most considerable in volume and of most importance as regards its influence upon contemporary and later medical opinion is the work of Klein (1888, 1889, 1890). This investigator *inoculated* eight cats subcutaneously and two intravenously with pure cultures of diphtheria bacilli. Four were unaffected but the other six showed swelling at the site of inoculation followed by death after five to eleven days. Postmortem all the fatal cases showed extensive haemorrhagic oedema locally and certain special kidney changes characterised by enlargement of the whole organ, almost the entire cortex being grey and fattily degenerated, the medulla appearing by contrast much congested. Microscopically the uriniferous tubules were swollen and the epithelium showed extensive fatty degeneration or broken down into a granular debris. Klein describes this condition of the kidney as extremely characteristic of diphtheria disease in the cat. The condition of the supra-renal capsules is not mentioned.

He also caused death or paralysis in two cats by injecting diphtheria bacilli direct into the trachea by a syringe pushed through the anterior wall.

One kitten and two cats fed with agar cultures in milk remained well, but two other cats fed on three separate occasions became very thin, although showing no other symptoms, and were killed three and four weeks after the start of the experiment. Both showed livers much enlarged, in one the kidneys



were large with white cortex, in the other the kidneys were not conspicuously large but their cortex was pale with a few whitish patches. Not the slightest bacteriological proof is given or evidence supplied of the presence of any clinical or pathological features of diphtheria but Klein remarks "From these experiments it is seen that by *repeated* feeding with cultures of diphtheria bacilli distributed in milk, unquestionable diphtheria disease can be produced in the cat." Klein also laid stress upon certain cases of accidentally acquired diphtheria (so called) in cats. The symptoms were an acute catarrhal affection of the conjunctiva and of the respiratory passages. The diagnosis of diphtheria was based upon the pathological appearances of the kidney and the appearance in one of the cats of a grey membrane covering the lower part of the larynx and upper part of the trachea in which "the microscope showed crowds of diphtheria bacilli as smaller or larger groups in the necrotic mucous membranes." Diphtheria bacilli were also demonstrated in the bronchial and tracheal exudation of another cat. Nine months later a somewhat similar outbreak occurred with similar pathological lesions and the presence of bacilli morphologically like diphtheria bacilli.

Klein records his examination of three cats sent to him from different sources and all associated with human cases of diphtheria. All three cats had been ill. The kidney condition which Klein records as typical of diphtheria was found in each case but no diphtheria bacilli were cultivated. On the basis of the pathological appearances and their likeness to the conditions induced in cats by inoculation with Klebs-Löffler bacilli Klein considered these were cases of cat diphtheria.

Renshaw (1885) in 1874 administered diphtheric membrane from human cases to a number of cats. Most died after illness and Renshaw reported finding diphtheritic membrane lining the fauces, bronchial tubes, etc. No evidence was adduced that the lesions were due to diphtheria bacilli and not associated with pathogenic cocci, etc.

Welch and Abbott (1891) inoculated into the trachea of a half-grown kitten a platinum loopful of a pure culture of the diphtheria bacillus. The animal died on the third day with the production of a diphtheritic membrane, containing diphtheria bacilli, in the trachea and larynx. No other noticeable lesion was found "unless it be a greater degree of fatty metamorphosis of the renal epithelium than is normal in kittens."

The above constitutes the only experimental work I have been able to trace.

Evidence is also available on the epidemiological side in the form of instances in which it is reported that the disease has been transmitted to human cases through the agency of cats. Without making a thorough search of the literature I have collected particulars of a number of such cases.

Turner (1886) appears to have been the first to report the association of illness amongst cats with cases of human diphtheria and he drew attention to their presence in a number of small outbreaks. The evidence was entirely

circumstantial and consisted of the concurrent presence of human cases with cats ill with such symptoms as swelling of the neck, foul discharge from the nostrils, eye inflammation, etc.

Bruce Low (1888) reported a similar relationship in an outbreak at Enfield.

Willams (1895) reported that in the same house as a case of human diphtheria three cats were taken ill with wasting, loss of appetite, inability to swallow, cough and expectoration and all three died. One cat was sent to Klein for examination who reported that there was extensive disease of the lung not uncommon amongst cats.

Gwynn (1893) reported at Hampstead the association of a case of human diphtheria with an ailing cat suffering from a bad swollen throat. Later it showed an unhealed abscess in the vicinity of the throat. No bacteriological examinations were made.

Dowson (1895) examined a cat which became ill in a house in which a child had died 14 days earlier from diphtheria. Cultures from the lung gave a pure culture of an organism morphologically resembling the diphtheria bacillus which was not further investigated.

Symes (1896) mentions a kitten which was in close and constant contact with a fatal case of diphtheria and which subsequently was attacked with vomiting and diarrhoea with slight swelling of the neck and with patches of yellow exudation on the fauces. These patches extended, the cat became much emaciated and died. Unfortunately the cat was buried before bacteriological examinations could be made.

Barras (1905) reports that in two cases of diphtheria in Govan a history of illness affecting the cats of the families attacked was ascertained. The throats of two of these cats were examined in the laboratory and micro-organisms were cultivated on artificial media which were found to correspond in every respect to those of the human diphtheria bacillus. No particulars are given of the tests employed for the identification nor are any details furnished as to the degree or nature of the illness from which the cats suffered. Barras also mentions another cat in Govan—a stray cat—which had obtained a home in one of the families affected and which was found to be suffering from post-diphtheritic paralysis of the legs. Apparently no bacteriological examinations were made.

Porter (1908) records a number of cases of diphtheria in a household containing two petted and favourite cats. The cats were not ill. Swabs were rubbed over the fur of each cat. One yielded negative results but smears from the culture from the other cat showed a considerable number of organisms identical in appearance with the Klebs-Löffler bacillus both when stained by methylene blue and by Neisser's method. Porter was unable to isolate the organism in pure culture.

Mapleton (1913), in connection with an outbreak of diphtheria amongst the children living in the Cottage Home, Newton Abbot, reported that there were "three cats in this Home in somewhat intimate association with the chil-



dren and a bacteriological examination of these proved that two of them were infected." Further inquiries showed that the examinations made were ordinary swabbings grown on blood serum and that the bacilli were not isolated in pure culture nor were any animal inoculations made. In this case the cats were not definitely ill although one was obviously not in health and were not suspected of having conveyed any infection. They were examined to prevent the possibility of their carrying infection elsewhere.

Webb (1914) reported an interesting outbreak at Leigh (Lancashire) amongst cats to which his attention was directed in connection with a case of diphtheria in a child. In the house with this human case the cat had been ailing for more than two weeks, had a peculiar cough, could not miaow properly, refused food, was very thin and could hardly move. During the previous week a cat next door had died presenting the same symptoms and a cat across the way had also died with the same symptoms.

The throat of the cat in the house with the diphtheria case was in a dirty sloughy condition and cultivations showed "a bacillus having the characteristics of the Klebs-Löffler bacillus." The organism was not isolated in pure culture and no cultural or inoculation tests were carried out. Webb states that five other cats in the street were found to be ailing and were destroyed.

Priestley (1915) investigating a number of cases of diphtheria at the Stockwell Orphanage found nine cats in that institution. All were bacteriologically examined although none of them were apparently ill. He reports "three of them in the boys' department and one belonging to the girls' department carried the diphtheria bacillus." In a personal communication he was kind enough to inform me that the bacilli were morphologically and culturally undistinguishable from the true Klebs-Löffler bacillus. No inoculation experiments were carried out nor is any information available as to what cultural tests were employed but apparently they were not isolated in pure culture and then tested.

The significance of the above data will be considered after my own experiments have been recorded.

#### THE AUTHOR'S INVESTIGATIONS.

These have been along three lines of inquiry:

A. The bacteriological examination of the throat and nose of healthy cats not associated with any cases of human diphtheria.

B. The bacteriological and pathological examination of cats associated with human cases of diphtheria.

C. Experimental investigations with kittens.

##### A. EXAMINATION OF HEALTHY CATS.

Eight cats and 12 kittens were examined, the throat being examined in every case while for most of the kittens and for a few of the cats the nose was also swabbed. One cat was examined twice. The method of examining

consisted in taking one or more swabs in the ordinary way and then charging at least three blood serum tubes and one agar tube in series without recharging. The usual order was 1 serum, 1 agar, 1 serum, 1 serum tube, the object being to obtain considerable dilution of bacteria on the last two serum tubes so that discrete colonies could be obtained. In some cases pea-flour trypt-agar plates, a medium upon which diphtheria bacilli grow well, were also inoculated. All the 12 kittens failed to show any bacilli which at all resembled diphtheria bacilli.

The eight cats were very different. Swabs from three of them showed no bacilli which might be taken for Klebs-Löffler bacilli. The results from the other five were as follows. The characters refer to films stained by methylene blue.

(1) Short bacilli, well marked beading chiefly at ends but some with central staining. Some curved and longer forms very closely resembling Klebs-Löffler bacilli. Bacillus isolated in pure culture.

(2) A number of bacilli present closely resembling Klebs-Löffler bacilli, thin, curved some thicker at ends and a few, but not the majority, showing polar staining. Do *not* show granules when treated with acetic acid. Isolated in pure culture.

(3) A large number of bacilli present resembling Klebs-Löffler bacilli. Curved, beaded but not definitely clubbed. No dark blue granules when treated with acetic acid. A bacillus resembling this organism isolated in pure culture.

(4) Numerous bacilli present which are curved, thin and beaded. Not thickened at ends but closely simulate Klebs-Löffler bacilli. Isolated in pure culture.

(5) A very mixed growth but contains a number of bacilli which very closely resemble true diphtheria bacilli being long, curved bacilli with granules at end but not in the middle. Granules show up well when the films were treated with acetic acid. Differ from typical Klebs-Löffler bacilli in being rather more uniform in size and the absence of clubbing. Was unable to isolate in pure culture.

No. 2 is of particular interest as it was the cat in a large girls' school. This cat was re-examined exactly three months later. = 2 *a*.

(2 *a*) Many bacilli closely resembling Klebs-Löffler bacilli present, being curved, and granular, thicker at one end and showing dark blue granules when the film was treated with acetic acid. Isolated in pure culture.

The bacilli isolated from cats (1), (2) and (3) were all alike so far as they were culturally investigated. They morphologically resembled true diphtheria bacilli less when isolated in pure culture although still superficially resembling it. Culturally they were distinct, the agar and blood serum growths being definitely yellow or yellow-white in colour while they produced no acid in glucose media and in litmus milk produced definite alkali after four to five days' growth.

The bacillus from cat (2 *a*) was extremely like the true diphtheria bacillus



both morphologically and culturally. It produced acid in glucose broth and grew like the diphtheria bacillus in milk. It was only distinguished so far as it was tested by the blood serum colonies having a distinct yellow tinge. Morphologically it was indistinguishable. It was non-pathogenic to a guinea pig.

It may be added that the films from the serum cultures made direct from the swabs were shown to the county bacteriologist in the laboratory who was examining several thousand suspected diphtheria swabs every year and he as well as myself was unable to distinguish the films from those from swabs from human throats containing true diphtheria bacilli.

We have therefore the striking fact that two experienced bacteriologists were unable to distinguish with any certainty these bacilli in mixed smears from true diphtheria bacilli although in several instances we were in some doubt and could not pronounce them typical bacilli since minute differences were apparent. In several cases the resemblance was extremely close. All of them however (with the possible exception of the strain from cat (2*a*)) were definitely not true diphtheria bacilli. Present in 66 per cent. of the adult cats it is interesting to note their entire absence from all 12 kittens.

#### B. EXAMINATION OF CATS ASSOCIATED WITH HUMAN CASES.

Although through *Public Health* medical officers of health were specially asked to send me such cases, none reached me in this way and the few cases investigated were all obtained through my personal efforts. Four were investigated in 1916 and one in 1919.

*Case 1.* Case of severe diphtheria in a house. A favourite cat much in the room with this case and with two children who suffered from severe colds but whose throats showed no diphtheria bacilli when swabbed.

The cat showed no signs of sore throat or other illness. Swabs from its throat were implanted on several blood serum tubes in series. The films showed numerous bacilli which in the primary films could not be distinguished from true diphtheria bacilli either by myself or the laboratory bacteriologist. A short form with marked polar staining. Isolated in pure culture the bacillus decidedly less closely resembled the Klebs-Löffler bacillus morphologically, while the yellow colonies and absence of acid production in glucose clearly showed it was not that organism.

If morphological appearances from the mixed growth swab had been relied upon undoubtedly this cat would have been classed as infected with diphtheria bacilli and would probably have been credited as the source of the infection.

*Case 2.* A human diphtheria case (a woman of 21) in a house. The house cat came into close association with this woman who was very fond of the animal. The cat was said to have been ailing for some days before the case was notified but no very clear particulars as to symptoms were forthcoming.

The cat when examined showed no local or general symptoms and the bacteriological examination of the throat swab showed a few bacteria suspiciously like diphtheria bacilli but nothing more definite.

*Case 3.* The cat in the house was closely associated with a case of human diphtheria. The animal remained throughout quite well and the cultures from the swab from its throat showed no bacilli at all like true Klebs-Löffler bacilli.

*Case 4.* A boy of eight years developed diphtheria on Nov. 22nd, 1916. The house cat was said to be "off its food" for about a week before that date and subsequently showed difficulty of swallowing as if it had a sore throat. The patient was very fond of this cat and fondled and nursed her regularly.

The cat was swabbed Dec. 6th. The films made from the blood serum cultures showed a few clusters of bacilli which morphologically rather resembled diphtheria bacilli of the short type. They were slightly curved, beaded and thicker at one end but rather thick compared with the true organism. Impossible to exclude as not Klebs-Löffler bacilli. They were only present in very small numbers and could not be isolated in pure culture. I was fortunate in being able to secure the cat and re-swabbed it in the laboratory but the cultures from this swab failed to show any bacilli suspicious of Klebs-Löffler bacilli.

The cat was killed and a postmortem examination made. There were no pathological lesions, the kidneys and other organs being quite healthy. Further swabs from the back of the throat and from the upper part of the trachea showed culturally no bacilli resembling diphtheria bacilli.

It may be mentioned that the cat when the first swab was taken and when later on examined at the laboratory showed no symptoms at all of ill health.

*Case 5.* A child, aged five, was removed to the isolation hospital suffering from diphtheria the diagnosis being bacteriologically confirmed. The child was said to have been ailing for at least ten days before removal. An elder sister (16 years) also had a sore throat before the notified case and was possibly the source of infection, but when swabbed the day the notified case was removed to hospital showed no diphtheria bacilli. According to the mother of the case the house cat was ill for 3-4 weeks before the child of five was notified, refusing to eat and with some discharge from the nostrils and mouth. The cat however gave birth to some kittens soon afterwards and then recovered and was quite well at the time of the removal of the case to hospital. The cat was brought to the laboratory April 16th, *i.e.* six days after the removal to hospital of the case, and swabbed. It showed no signs of any illness or nasal discharge. The animal was killed and postmortem showed no membrane in the throat or any lesions anywhere.

The films from the blood serum cultures made showed (mixed with abundant cocci) bacilli which were beaded, curved and thicker at one end and which could not be distinguished from diphtheria bacilli. With great difficulty this organism was isolated in pure culture and investigated. Morphologically



it could not be distinguished from the diphtheria bacillus and the blue granules after treatment with acetic acid were very marked. Culturally it grew like diphtheria bacilli on ordinary agar and blood serum but unlike our ordinary diphtheria strains more abundantly on the former than on the latter. Indeed it did not grow well on blood serum. It produced acid in glucose broth but none in lactose or mannite broth while it produced a trace of acid in saccharose broth.

To test its pathogenicity a guinea pig was inoculated subcutaneously from a broth culture reinforced by the growth on a tube of pea-flour trypt-agar (*i.e.* a very heavy dose) but the animal was unaffected.

The cultural characters agree with the diphtheria bacillus for the most part and this may possibly have been a non-pathogenic Klebs-Löffler bacillus.

### C. EXPERIMENTAL INVESTIGATIONS WITH KITTENS.

If, as has been so often asserted, cats suffer from diphtheria or even if it be merely advanced that they act as carriers of the diphtheria bacillus in their throat or nose, it should be possible to infect them artificially and set up either condition.

The possibility of this was tested in a long series of experiments. Kittens were used in preference to adult cats as both more easily handled and as likely to be more susceptible to infection than the grown up animals. In every case the throat and in nearly all cases the nose of the animals were swabbed before the experiments started to ascertain if diphtheria-like bacilli were present or absent.

The kittens were all kept in separate cages and very carefully examined for any signs of ill health. Weight observations were recorded but temperature testings were not found very helpful. The kittens were usually from four to six weeks old when the experiments started.

*Exp. 1.* Throat swabbed with, as far as possible, all the growth of a blood serum culture of *B. diphtheriae* transferred on a sterile swab. Immediately re-swabbed in the same way with a second blood serum culture of another strain.

Throat swabbed after three and seven days and several blood serum tubes inoculated. No diphtheria bacilli found. Animal showed no signs of any illness, quite lively and gained in weight. No local throat-lesions.

*Exp. 2.* Throat of same kitten re-swabbed a month after onset of *Exp. 1* with as far as possible the whole of a blood serum culture made direct from a swab taken from the throat of an acutely ill case of diphtheria. This culture showed about half the colonies *B. diphtheriae* and half streptococci and it was used as possibly the mixed growth might favour infection. Animal remained quite well and lively and a swab taken six days after the inoculation showed no diphtheria colonies on the several blood serum tubes inoculated.

Animal killed eight days after the second inoculation. Postmortem all the organs were healthy and sections of kidney and supra-renals showed no abnormalities. No trace of any lesions in the throat.

The virulence to guinea pigs of these three strains was not tested.

*Exp. 3.* Kitten about four weeks old fed on two consecutive days with milk containing the whole of two blood serum growths of two different strains of *B. diphtheriae*, one of which was fully virulent to a guinea pig. Animal showed no symptoms whatever and the throat swabbed six days after the first feeding yielded no diphtheria bacilli.

*Exp. 4.* Throat of the same kitten swabbed with, as far as possible, the whole of two blood serum cultures of *B. diphtheriae* (plus a small number of staphylococcus colonies) grown direct from the throat of an acute case of diphtheria. Throat swabbed seven days later showed no diphtheria bacilli. Animal showed steady increase in weight and no local or general symptoms.

Kitten killed 22 days after onset of the feeding and 15 days after the throat swabbing. No trace of membrane on throat, trachea or larynx. Internal organs normal and sterile. Sections of the kidney and supra-renals showed no pathological changes except some engorgement of the kidney blood vessels.

*Exp. 5.* Throat of a kitten about five weeks old swabbed with the whole of a blood serum growth from a fifth strain (strain B). This blood serum growth was direct from a swab from an acute case and showed *B. diphtheriae* in almost pure culture. Swabs from the throat taken 4 and 10 days after inoculation showed no diphtheria bacilli. The animal increased in weight and exhibited no signs of ill health.

*Exp. 6.* Thirteen days after *Exp. 5* started this kitten was injected subcutaneously with 2 c.c. of a five days old (trypsin pea-flour) broth pure culture of the same strain (strain B) of diphtheria bacillus. The animal was obviously ill within 24 hours of the injection and remained very quiet. The temperature which for the two days before the experiment had fluctuated between 99° and 100° rose to 104° F. the morning and also the evening after the inoculation. It remained between 102° and 104° for seven days, then rapidly dropped to below 100° C. and was 96° on the ninth day when the animal was moribund and was killed. The day before death very definite paralysis of the hind legs was observable. The postmortem examination showed no oedema or inflammation at the site of inoculation, no fluid excess in the thoracic cavity. The internal organs, apart from the kidneys and supra-renals, appeared healthy. Both kidneys were greatly enlarged and in section showed marked cloudy swelling of the cells of the cortex and medulla with enlargement of all the blood vessels. The supra-renals showed a condition of acute inflammation with dilated engorged blood vessels and swollen, turbid and indistinct cells in both cortex and medulla.

This experiment is important as showing that the same strain which was incapable of infecting or affecting the kitten by means of the throat was highly pathogenic when injected into the same animal.

*Exp. 7.* A rather older kitten (about two months) and weighing 830 grms. used for a combined throat swabbing and feeding experiment.

Throat swabbed with the whole of a blood serum culture of another



*B. diphtheriae* strain (strain J). Throat examined after two, five, and nine days showed no diphtheria bacilli.

Five days after the third inoculation fed with milk in which strain J had been grown for 24 hours fortified by the whole of a 24 hours growth on blood serum of the same organism.

Feeding with the same massive dose repeated on the following three days. Throughout the animal remained quite well and gained steadily in weight.

*Exp. 8.* The same kitten some weeks later injected subcutaneously with 1.8 c.c. of a two days glucose broth culture of strain J. The kitten showed a slight rise of temperature but no other symptoms and increased in weight. Killed 14 days after the injection showed no macroscopic or microscopic lesions. The growth in the broth culture was scanty and the kitten weighed when inoculated 1020 grms. so the dose was probably inadequate and this experiment hardly indicates the strain was non-virulent.

*Exp. 9.* The nose of a young kitten about four weeks old inoculated heavily with a pure culture on blood serum of *B. diphtheriae* (strain W) transferred on a thick blunt platinum needle. Previous swabbings of nose (both sides) and throat showed absence of any diphtheria bacilli-like organisms.

Nose swab examined 24 hours after the inoculation failed to show any diphtheria bacilli when grown on blood serum tubes. Both sides of the nose then again inoculated with *B. diphtheriae* in the same way as before and each nostril examined after 48 hours and eight days showed no diphtheria bacilli.

The animal remained perfectly well with no nasal discharge or any symptom.

*Exp. 10.* It may be argued that the failure to infect the kittens by the throat was due to the smooth healthy mucous membrane failing to afford a nidus for growth and the following and some other experiments were devised to deal with this objection.

Thirteen days after the onset of *Exp. 9* the throat of this kitten was well painted with a 20 per cent. solution of silver nitrate. Examined 24 hours later there was some reddening of the throat and superficial ulceration of the roof of the palate. Throat re-painted with the silver nitrate solution. After a further 24 hours there was definite redness with much mucous and some whitened areas on the palate. The throat was then inoculated with, as far as possible, the whole of a two days old blood serum culture of *B. diphtheriae* (strain W). Swabs from the throat 24 hours and 48 hours after the *B. diphtheriae* implantation when grown on blood serum showed no diphtheria organisms. The animal remained fairly well but was quiet and did not put on much weight but gradually recovered completely.

*Exp. 11.* Eight days after the start of *Exp. 10* this kitten was injected subcutaneously with 2 c.c. of a 24 hours broth culture (pea-flour trypt broth) of the same *B. diphtheriae* strain (strain W). Animal obviously ill 24 hours later. After 48 hours very ill, quiet and refused food. Three days about the same and after four days appeared better but died on fifth day.

Postmortem the animal showed the signs noted above as present in the other injected kitten except that the reddening of the supra-renals was trifling or absent. They showed however inflammation signs when sections were made and stained. The kidneys were especially engorged and enlarged. Exps. 10 and 11 show that this strain while highly pathogenic when injected was unable to establish itself upon the throat of the same animal even when a definite unhealthy and pathological nidus was provided.

*Exp. 12.* The throat of a young kitten swabbed with as far as possible the whole of a two days old blood serum growth of *B. diphtheriae* (the virulent strain W being used). Nose (left side) also inoculated from another serum culture of the same organism. No diphtheria bacilli found in the throat when examined after 24 and after 48 hours. Diphtheria bacilli found in the nose at the end of 24 hours but not after 48 hours or subsequently. Animal remained quite well.

*Exp. 13.* Nose and throat of another quite young kitten inoculated heavily from another *B. diphtheriae* strain (strain V). Both nose and throat were free from diphtheria bacilli when examined after 24 and 48 hours.

*Exp. 14.* This same kitten was then fed on eleven occasions over 14 days with a pea-flour broth culture of *B. diphtheriae* (strain W). This broth gave abundant growth and over this period as much as 100 c.c. of broth culture was administered. No illness or symptoms of any kind. The animal steadily increased in weight and the postmortem examination 3½ weeks after the commencement of the feeding showed no abnormalities.

*Exp. 15.* The hard palate (as far back as possible) of another young kitten was scarified by a scalpel making a number of superficial lesions. A *B. diphtheriae* (strain W) blood serum culture transferred on a sterile swab was then at once well rubbed into these superficial lesions. The animal remained quite well and examined 24 and 48 hours after showed no membrane or other lesions nor could diphtheria bacilli be cultivated from the swabs taken although a good many tubes were used.

*Exp. 16.* Throat of a young kitten swabbed with a strong solution of silver nitrate. Examined 24 hours after there was a well marked slough. The throat was inoculated with a blood serum culture of diphtheria bacilli. The animal was very ill and as it was in pain was killed. Here the throat treatment was too severe but swabs after 24 hours showed no diphtheria bacilli and postmortem there was no evidence of any true membrane although sloughs over the hard palate. Films from throat and trachea showed no bacilli like *B. diphtheriae*.

*Exp. 17.* The nose of another young kitten inoculated heavily with *B. diphtheriae* (strain W). Nose swabs taken previously showed no bacilli at all like diphtheria organisms. Nose examined after 24 hours and after 48 hours showed diphtheria bacilli colonies on the blood serum tubes and apparently more abundant after the longer period. None found after four and five days. Unfortunately not examined after three days. The animal



remained perfectly well, gained in weight, had no nasal discharge and post-mortem showed no lesions.

*Exp. 18.* Nose of another kitten inoculated heavily with two separate strains of *B. diphtheriae* (strains W and V).

After 24 hours diphtheria bacilli readily grown from the nose. Found but less readily after 48 hours. Examined after four days the bacilli were found in the cultures but with great difficulty and could not be found at all after five and six days. The kitten remained perfectly well throughout, increased in weight and showed no nose discharge or other local lesions.

*Exp. 19.* The nose of another kitten inoculated heavily with another *B. diphtheriae* strain (strain R) isolated a day or two previously from an acute case of diphtheria. After 24 hours a few diphtheria bacilli were grown from the nose but none after 48 hours, four and five days. Animal remained perfectly well without any local or general symptoms.

#### REMARKS ON THE EXPERIMENTS.

The results obtained with these young kittens are exceptionally uniform and concordant. It was found impossible to infect them by throat swabbing although very massive doses were invariably used, as many as 10 different strains employed and all of them were quite recently isolated from acute human cases of diphtheria. Further, four of the strains used were proved to be of high virulence and two definitely killed, when subcutaneously injected, young kittens they were unable to infect by natural channels of entry. The same failure to infect these animals with diphtheria or any local lesion was experienced when mixed cultures direct from human throats were used or when an artificial nidus for local growth was provided by chemical or mechanical means. Not only did the bacilli not infect but they failed to survive even and although such massive doses were inoculated they invariably disappeared after as short a period as 24 hours.

The implantations of vast numbers of diphtheria bacilli into the nasal cavities were equally ineffective in setting up any local or general lesions and (what I did not expect to find) were unable even to survive beyond a very short period. In only one experiment was there possible evidence of increase and only in one instance could the most diligent examination trace their persistence beyond four days.

The feeding experiments were also unsuccessful and the kittens consumed vast numbers of virulent diphtheria bacilli without the slightest evidence of harm.

These experiments so far from supporting the view that diphtheria is a naturally occurring disease of cats suggests that the secretion from the mucous membranes of the cat are peculiarly unfavourable to the growth of the diphtheria bacillus and will not even permit it to remain as a saprophyte.

## CRITICAL CONSIDERATION OF THE AVAILABLE FACTS.

There are two separate possibilities. On the one hand there is the suggestion that cats themselves may be affected with disease due to their infection with the diphtheria bacillus, and which therefore may be appropriately designated as cat diphtheria, while on the other hand we have to consider the possibility that these animals may act as carriers of diphtheria infection by harbouring the bacilli in their nose or throat or by their fur becoming infected.

As regards the first possibility it must be accepted from the work of Klein and others, corroborated by my own experiments, that cats are not immune to the toxins of *B. diphtheriae* and that when these are introduced under the skin or by other means such as direct injection into the trachea fatal results may occur with the development of pathological lesions not dissimilar from those which are observed when guinea pigs or other animals are similarly injected. This of course no more proves or even suggests that cats suffer from diphtheria than it does that guinea pigs so suffer or the latter animals from typhoid fever because they succumb to the injection of typhoid bacillus toxins.

A study of the experimental data in favour of cats suffering from diphtheria detailed in the first part of this paper shows that the evidence is extremely weak and unconvincing and does not warrant the interpretation placed upon it. My own considerable series of experiments, testing by many different ways the possibilities of natural infection, are in direct opposition to the widely accepted view that cats can suffer from a kind of diphtheria.

The evidence which has been adduced from the association of human diphtheria cases with cases of illness amongst cats whose throats are said to show diphtheria bacilli must be regarded as *valueless* in almost every case, for the following two reasons.

In all the instances that I have been able to find, with the exception of one case recorded by Barras and possibly Case 5 of my series, the diagnosis rests entirely upon the presence in the throat of the cats of bacilli morphologically like *B. diphtheriae*. Barras does not mention the steps he took to identify his bacillus and one cannot exclude the possibility of it being a pseudo-diphtheria bacillus. The results recorded above show that in *normal* cats no less than 66 per cent. showed bacilli which without isolation and study in pure culture I was unable to exclude as not true diphtheria bacilli. In my series of cats associated with cases of human diphtheria both Cases 1 and 4 would without doubt have been classed and accepted as proved cases of diphtheria in cats although a more complete investigation quite refuted this assumption.

The second reason is that the fact that the cat or cats were ill does not supply even *prima facie* evidence for suggesting that the human and cat conditions were related.

It is recognised by veterinary authorities that cats suffer from illness with symptoms which in some respects are analogous to those of human diphtheria but which are certainly not cases of diphtheria or due to *B. diphtheriae*.



Gray (1896), for example, states that the so-called diphtheria in the cat is a contagious disease of the cat, characterised by the presence of diphtheritic membranes on the fauces, pharynx or larynx and due to some micro-organism not yet determined. The disease has a mortality of quite 90 per cent.

Gofton (1913) says that the kidney condition described by Klein as pathognomic is an exceedingly common condition of the cat's kidney. He adds "affections presenting diphtheritic characters are met with in the cat and occur independently of human diphtheria, but the bacteriological investigation of these affections has up to the present always resulted in a failure to demonstrate the presence of the human bacillus." With a prevalent disease such as human diphtheria and a not very rare illness of cats with the above symptoms it is obvious that the two will be associated together in a certain proportion of cases. I investigated my first cases of supposed cat diphtheria (with negative results, not recorded here) just over 20 years ago and I have been on the look out for associated cases ever since and undoubtedly the association of the two conditions is infrequent. In fact we shall probably be correct in affirming that the two diseases are not associated together in any higher proportion than the relative frequency of the two conditions mathematically postulates. There are therefore no grounds for the assumption so commonly made that when the two conditions do happen to coincide we should assume a relationship of cause and effect between the two.

The interesting cases of illness in cats recorded by Webb (*loc. cit.*) are clearly examples of this infectious disease in cats and there is no justification for dragging in the human factor to account for them.

My experimental work affords no support to the suggestion that the cats may act as carriers by the bacilli living in their throat or nose. On the contrary it would appear that the mucous membranes of these animals are particularly inimical to these bacilli and that even under the most favourable circumstances they are unable to multiply or even survive for more than a trifling period. That the fur of these animals may be infected with these bacilli is of course quite a possibility and there is some experimental evidence in its favour (*e.g.* Remlinger, 1906) but in view of the low resistance powers of *B. diphtheriae* this is probably an unimportant factor in the spread of the disease.

Summing up the matter I am of opinion that the common and widely accepted view that cats can suffer from a naturally acquired disease caused by the diphtheria bacillus is entirely without foundation.

The reported cases of such an association are based upon insufficient examination and differentiation of the bacilli due to a failure to realise that a large proportion of healthy normal cats contain in their throats bacilli which closely resemble and are difficult to distinguish from the true *B. diphtheriae*.

## REFERENCES.

- BARRAS, W. G. (1905). *Lancet*, II. 1869.  
 — (1906). *Public Health*, XVIII. 709.  
 DOWSON, W. (1895). *Report of Medical Officer of Health, Bristol*.  
 GOFTON, A. (1913). In Hoare's *System of Veterinary Med.* I. 310.  
 GRAY, H. (1896). *Journ. of Comp. Path. and Therap.* IX. 46.  
 — (1913). In Hoare's *System of Veterinary Med.* I. 317.  
 GWYNN, E. (1893). *Brit. Med. Journ.* II. 1013.  
 KLEIN, E. (1888). *Report of Medical Officer, Local Government Board*, 435.  
 — (1889). *Ibid.* 156.  
 — (1890). *Ibid.* 229.  
 LOW, BRUCE (1888). *Ibid.* 131.  
 MAPLETON, H. B. (1913). *Annual Report Newton Abbot Urban District Council*, and personal communication.  
 PORTER, A. E. (1908). *Brit. Med. Journ.* I. 622.  
 PRIESTLEY, J. (1915). *The Medical Officer*, June 5th, 253, and personal communication.  
 REMLINGER, P. (1906). *Revue Scientifique*, Dec. 29th, 801. Summary. *Brit. Med. Journ.* 1907, I. 552.  
 RENSHAW, C. J. (1885). *Brit. Med. Journ.* I. 12.  
 SYMES, J. O. (1896). *Ibid.* I. 1385.  
 TURNER, G. (1886). *Report of Medical Officer, Local Government Board*, 307.  
 WEBB, F. (1914). *Journ. Royal San. Inst.* XXXV. 63, and personal communication.  
 WELCH and ABBOTT (1891). *Johns Hopkins Hosp. Bull.* II. 25.  
 WILLIAMS, W. (1895). *Brit. Med. Journ.* II. 74.



## A NOTE ON "DEFENCE RUPTURE" AND THE ACTION OF ELECTROLYTES.

BY W. CRAMER AND W. E. GYE.

*Imperial Cancer Research Fund, London.*

THE etiology of tetanus and gas gangrene presents a biological problem of an unusual nature. When the organisms which cause these diseases are deprived of toxin, by washing in tap-water saline or by washing and then heating, they are non-pathogenic to laboratory animals. In a recent investigation of the problem<sup>1</sup> we have shown that soluble ionisable calcium salts enable detoxicated *B. tetani*, *B. Welchii*, *B. oedematiens* and *vibrion septique* to exert their pathogenic powers. We concluded that calcium owes this property to its local action on the tissues.

Our conclusions have been criticised by Shearer<sup>2</sup>, who offers an alternative explanation of the facts. Shearer has shown that the unbalanced Na ion rapidly kills the meningococcus, and has brought forward evidence to prove that the pneumococcus and *B. anthracis* lose their pathogenicity when treated with a solution of pure NaCl in distilled water. His explanation of our work is that by washing in saline the gas gangrene bacilli are robbed of their pathogenicity which is restored by adding Ca. According to Shearer our experiments are examples of the interaction of bivalent and monovalent ions on the normal stability of the cell wall. We think our previous papers contain sufficient evidence to nullify Shearer's view of our work. The following experiments show it to be incorrect.

### EXPERIMENT 1.

A large tube of meat broth was inoculated with spores of *vibrion septique* which had been kept in tap-water saline at room temperature for 15 months. In 24 hours there was a vigorous growth with evolution of gas. The culture was kept at 37° C. for 48 hours and was then washed twice in Ringer's solution<sup>3</sup>. The final thick deposit of particles of meat and spores was heated in vaccine phials to 80° C. for 25 minutes. On the 3rd December, equal quantities of the spores were added to (A) 5 c.c. of Ringer, and (B) 2.5 c.c. of Ringer to which 2.5 c.c. of a 1 per cent. solution of CaCl<sub>2</sub> was added. Ten mice were then inoculated with suspension (A) and ten with suspension (B).

<sup>1</sup> *Proc. Roy. Soc. Ser. b*, xc. 513; also 6th Scientific Report, Imperial Cancer Research Fund.

<sup>2</sup> *Journ. of Hygiene*, xviii. 337.

<sup>3</sup> Composition: NaCl 0.77 %; KCl 0.024 %; CaCl<sub>2</sub> 0.0208 %.

*Action of Electrolytes*

	Inoculum	Dose and site	Result
(A) Mice 1 to 10.	Suspension of v. s. spores in Ringer.	0.5 c.c. right flank.	4 Dec. All alive and well. The animals remained in good health. On 10 Dec. each was injected with 0.5 c.c. of 1% $\text{CaCl}_2$ : 11 Dec. all were dead of gas gangrene.
(B) Mice 11 to 20.	Suspension of spores in equal quantities of Ringer and 1% $\text{CaCl}_2$ .	0.5 c.c. right flank.	4 Dec. 9 dead of gas gangrene; 1 alive and well. This animal remained in good health. On 10 Dec. it was injected with 0.5 c.c. of 1% $\text{CaCl}_2$ : 11 Dec. dead of gas gangrene.

## EXPERIMENT 2.

4th December. A tube of serum agar was inoculated with spores of *vibron septique* and the tube incubated anaerobically. 6th December a thick growth of a pure culture of *vibron septique*. The culture was taken off from the surface of the serum agar by means of a platinum loop and was emulsified in 1 c.c. of Ringer; 0.5 c.c. of the emulsion was added to (A) 3 c.c. of Ringer and 0.5 c.c. to (B) a mixture of 1.5 c.c. of Ringer and 1.5 c.c. of 1 per cent.  $\text{CaCl}_2$ .

Six mice were inoculated with the suspension in Ringer and six animals with the suspension in Ringer +  $\text{CaCl}_2$ .

	Inoculum	Dose and site	Result
(A) Mice 1 to 6.	Suspension of organisms in Ringer.	0.5 c.c. right flank.	7 Dec. Five alive and well. One dead of gas gangrene. The five mice remained in good health. 10 Dec. Inoculated with 0.5 c.c. 1% $\text{CaCl}_2$ . 11 Dec. All dead of gas gangrene.
(B) Mice 7 to 12.	Suspension of organisms in Ringer + $\text{CaCl}_2$ .	0.5 c.c. right flank.	7 Dec. All dead of gas gangrene.

## EXPERIMENT 3.

4th December. A culture of *B. Welchii* in meat broth was made.

5th December. Vigorous growth. Culture pure. 50 c.c. of culture centrifuged and broth pipetted off from deposit, which was then washed thrice with sterile Ringer. The final deposit was emulsified in 1 c.c. Ringer; 0.5 c.c. of the emulsion was added (A) to 3 c.c. of Ringer; (B) 0.5 c.c. to a mixture of 1.5 c.c. Ringer and 1.5 c.c. 1 per cent.  $\text{CaCl}_2$ .

The following inoculations were made:—

	Inoculum	Dose and site	Result
(A) Mice 1 to 6.	Suspension of <i>B. Welchii</i> in Ringer.	0.5 c.c. right flank.	All alive and well; the mice have remained in good health.
(B) Mice 7 to 12.	Suspension of <i>B. Welchii</i> in Ringer and $\text{CaCl}_2$ .	0.5 c.c. right flank.	All the mice died within 36 hours of gas gangrene.
(C) Mice 13 to 18.	Whole meat broth culture of <i>B. Welchii</i> .	0.25 c.c. right flank.	All mice died within 36 hours of gas gangrene.



## THE CONTAMINATION OF OYSTERS.

By A. T. NANKIVELL, M.D. (LOND.), D.P.H. (CAMB.),

*Medical Officer of Health, Poole,*

AND SERGT J. M. STANLEY, A.A.M.C.

(With 1 chart.)

### TOPOGRAPHY AND TIDES.

THE Harbour of Poole lies a few miles to the West of Bournemouth. It has an area approximately of 10,000 acres, but there is only a very narrow passage between its waters and the open sea. The width of this bottle-neck entrance is about 250 yards. The last published Admiralty chart gives the rate of the incoming tide through this narrow entrance at  $3\frac{1}{4}$  knots, and of the outgoing tide at 4 knots. The local opinion is that these official rates err on the moderate side. The rapid ebb and flow of the tides has an influence in the bacteriology of the oysters within the harbour.

The presence of the Isle of Wight to the eastward causes Poole Harbour and Bournemouth Bay to have four tides instead of the usual two. About three-quarters of an hour after the first high water the tide begins to ebb; but about  $2\frac{3}{4}$  hours later it is high again. The first flood tide begins  $4\frac{1}{2}$  hours before high water.

### OYSTERS.

For many years past the oysters dredged from Poole Harbour and from Bournemouth Bay have been contaminated. The reports of various Medical Officers have associated these oysters with cases of Enteric Fever. One of the present writers has known of Enteric Fever which occurred after eating Poole or Bournemouth oysters: no other possible cause of the disease could be ascertained in these cases. Samples of oysters have been taken infrequently and at irregular intervals in the past and examined bacteriologically: they were found by various bacteriologists to be polluted, more or less heavily, with *B. coli*, and sometimes with *Streptococci* and *B. welchii* (Table I). At the present time Poole oysters cannot be dredged except for relaying, and this ban upon them is of considerable financial loss to the district.

### THE PRESENT ENQUIRY.

Early in 1914 one of us (A.T.N.) was appointed to the post of Medical Officer of Health to the Borough and Port of Poole, and before the outbreak of war he had begun some research work on the subject of the pollution of

the harbour water and of the oysters, hoping by this to settle once and for all, by a thorough investigation, many details concerning the oyster pollution, which had been unsatisfactorily dealt with in the past by other observers in a partial manner and unconvincingly. This earlier research was necessarily interrupted, but on his return to the Borough the investigations were continued. The enquiry was directed towards:

1. The source of pollution of the oysters.
2. The nature of the infecting micro-organisms.
3. The discovery, if possible, of a suitable relaying place; and
4. In the absence of this, the effect of sterilisation by chlorinated sea-water.

Towards the cost of this research the Borough Council of Poole made a contribution of five pounds, and the Southern Sea Fisheries Board a grant of a similar amount. All the work referred to in this paper was done at the Poole Borough Laboratory; and we should like here to express our appreciation of the help given to us by our Laboratory Attendant, S. Marshall, who prepared most of our culture media, and aided us generally in the work.

#### TECHNIQUE.

Samples both of sea-water and of minced oysters were plated in neutral-red-bile-salts-peptone-lactose-agar. Both single strength and double strength were used—the latter for the plates of 10 c.c. and upwards. Of the sea-water we plated varying quantities from 0.1 c.c. to 15.0 c.c.; and examined in MacConkey Lactose tubes quantities from 20 c.c. to 100 c.c. We should like here to insist that plate cultivation gives more accurate results than can be obtained by the use of liquid media. If a 20 c.c. tube shows acid and gas it cannot accurately be estimated exactly how many lactose fermenters were present: if, however, four plates each of 5 c.c. are made, then the number of lactose fermenters can be seen and correctly counted. Gradually, as the work progressed, we used more plates and fewer tubes. Apart from this, our technique was that usually employed in the examination of samples of drinking water, as far as the sea-water was concerned. When dealing with the oysters we cleansed the outsides of the shells by thorough scrubbing under running water. The oysters were then opened with a sterilised knife, and the contents finely minced before removal from the concave shell. Each oyster was then emulsified in 100 c.c. of sterile saline. In dealing with oysters which were presumably polluted, plates of 0.5 c.c., 1 c.c. and 5 c.c. of the emulsion were made. When examining oysters after relaying we used six plates each of 5 c.c. and two plates each of 10 c.c. Saline emulsion corresponding to half an oyster was therefore plated and examined. On several occasions we checked our plate results by inoculating a series of MacConkey Lactose tubes.



## THE SOURCE OF POLLUTION OF THE OYSTERS.

The first oysters examined, taken from a bank just inside the harbour mouth, showed the presence of many lactose fermenters—about 700 per oyster, quite enough in our opinion to condemn them. (See Table I.) We had then to decide from what quarter this pollution came; did it originate within the harbour of Poole, or was it brought in from the outside?

Table I.

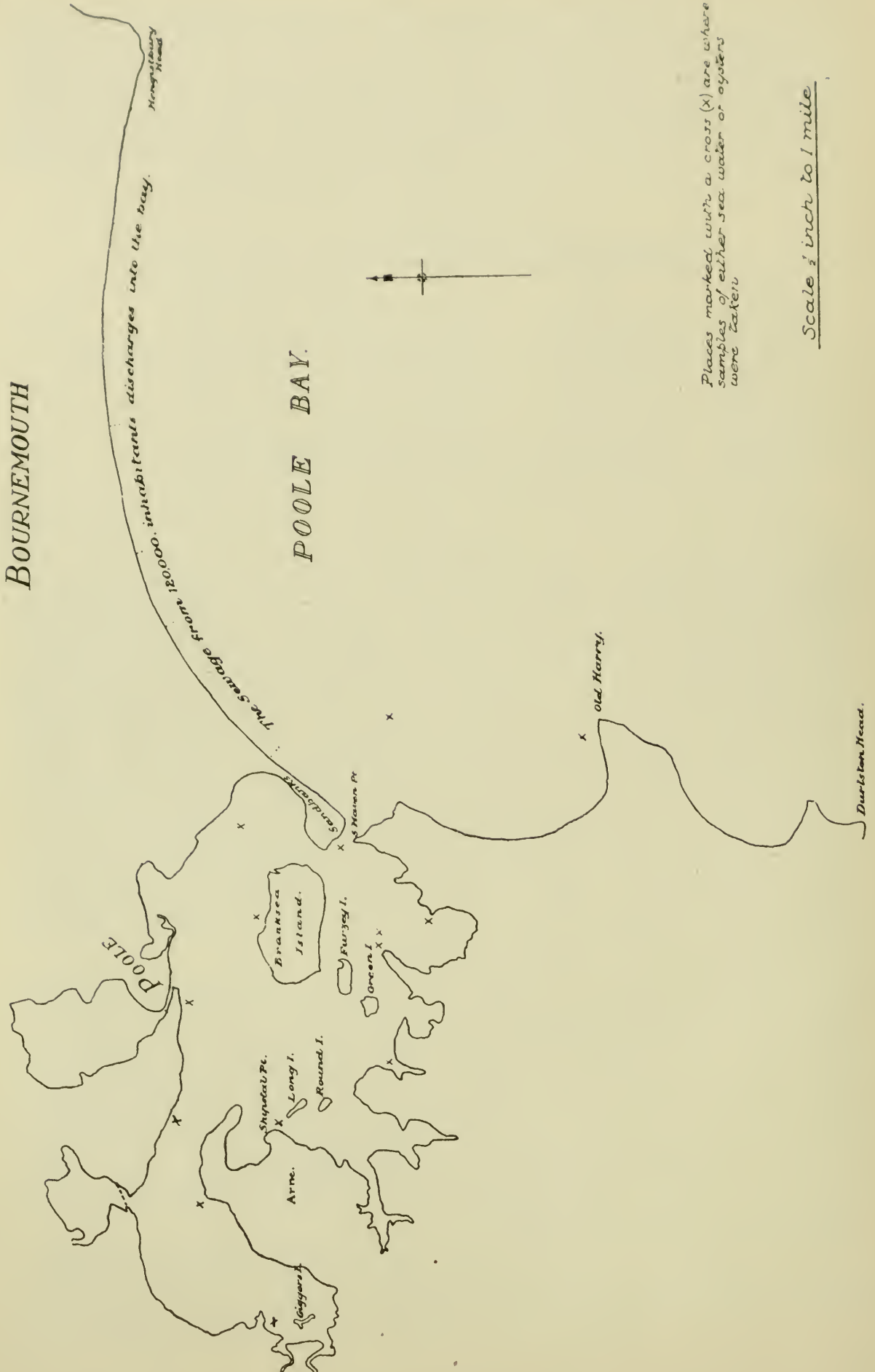
*Poole and Bournemouth Bay Oysters, 1914–1919.*

Date	Place	Bacteriological findings
May, 1914	Poole Harbour	700 lactose fermenters per oyster
June, 1914	Poole Harbour	<i>Streptococci</i> and <i>B. welchii</i> in $\frac{1}{100}$ of an oyster
June, 1914	Poole Harbour	1500 lactose fermenters per oyster
May, 1915	Poole Harbour	3000 lactose fermenters per oyster
Nov. 1915	Poole Harbour	<i>B. coli</i> and <i>streptococci</i> in $\frac{1}{5}$ c.c.
Sept. 1919	Poole Harbour	2400 lactose fermenters per oyster
Oct. 1919	Bournemouth Bay	1500 lactose fermenters per oyster

To settle this question we took half hourly samples of the water at the bottle-neck entrance to the harbour from Sandbanks Pier (see chart). The samples were taken throughout several tides. They were obtained in sterilised bottles from a depth of three feet below the surface by means of a specially designed plunger. The samples were sent by bicycle or motor-car to the laboratory four miles away, and were plated and tubed within a few hours of being taken. The first results were a surprise to us, and the experiment was therefore repeated over two other tides. The second series of plates however showed results which were generally constant—sufficiently so, at any rate, to prove without any doubt that the first incoming tide brought many lactose fermenters into the harbour; that on the second tide not so many were brought in, and that the water at outgoing tides was comparatively clean and free from micro-organisms (Table II). The pollution of the oyster beds therefore came in from the open sea; and not from the Town of Poole or from the rivers that flow into the top of the Harbour.

A knowledge of the local sewage outfalls and of the tides in the bay will explain how these faecal organisms can make their way in from the open sea. All the sewers of Bournemouth and of Poole discharge into the sea, and not into the harbour: the sewage is carried out in the ebb tide in the direction of the headland known as Old Harry. On arrival in this neighbourhood it is met by the strong incoming tide and pushed into Poole harbour. Subsidence of micro-organisms and certainly of macroscopical pieces of sewage takes place here, and this explains the finding that the water leaving the harbour is cleaner than that which comes in.

Having satisfied ourselves by these water samples, taken at Sandbanks, that the pollution came in on the first high tide, we next proceeded to ascertain how the water in different parts of the harbour was affected. We followed up



Places marked with a cross (X) are where samples of either sea water or oysters were taken

Scale  $\frac{1}{2}$  inch to 1 mile



the Main Channel taking samples at Salterns, at Hamworthy and off Russell Quay during different stages of the tide: these were infected, but, speaking generally, not so heavily as the water at Sandbanks. We found the same in the South Channel at Redhorn and at Goathorn, and in the Wych Channel opposite the Ower (Table II).

At these places we did not repeat our observations over several days, nor take so many samples—there seemed to be no need since none of the results were at variance with our earlier observations at Sandbanks.

IDENTITY OF MICRO-ORGANISMS.

We were not able to make a prolonged and thorough investigation of the lactose fermenting organisms which we isolated from the sea-water and from oysters. One of us (J.M.S.) had only four months to spend on non-military employment in this country before his return to Australia, and it was possible therefore systematically to examine only a small proportion of the organisms

Table II.

*Showing number of lactose fermenting organisms in 10 c.c. of the sea-water at various places at different stages of the tide.*

State of tide	Hours after low water	Sand-banks Pier	Sand-banks Pier	Sand-banks Pier	Salterns	Hamworthy	Russell Quay	South Channel	Redhorn	Goathorn	Wych	Ower	Shipstall
Low water	0	0	1	0	—	—	—	—	—	—	—	—	—
	$\frac{1}{2}$	0	2	1	—	—	0	—	—	—	—	—	0
Incoming tide	1	0	3	0	—	—	0	—	—	—	—	0	0
	$1\frac{1}{2}$	1	2	1	—	—	1	—	1	—	1	0	0
	2	14	0	12	10	—	0	—	1	—	3	0	0
	$2\frac{1}{2}$	38	6	20	12	10	20	—	0	—	10	0	0
	3	56	14	24	12	30	44	—	26	—	12	0	0
	$3\frac{1}{2}$	86	20	32	14	16	8	—	14	—	—	0	0
	4	100	48	48	26	—	70	—	—	—	—	0	0
1st high water	$4\frac{1}{2}$	82	12	8	2	—	15	2	—	—	—	—	0
	5	6	1	2	—	—	7	1	—	—	—	—	0
	$5\frac{1}{2}$	2	0	1	—	—	0	28	—	1	—	—	0
	6	0	0	0	—	—	0	10	—	1	—	—	0
	$6\frac{1}{2}$	0	0	0	—	—	0	5	1	4	—	—	0
	7	0	0	0	—	—	0	—	0	1	—	—	0
	$7\frac{1}{2}$	0	0	0	—	—	0	—	0	—	—	—	0
	8	0	0	0	—	—	1	—	1	—	—	0	0
2nd high water	$8\frac{1}{2}$	1	1	0	—	—	0	—	—	—	—	0	0
	9	1	0	1	—	—	3	—	—	—	—	1	0
	$9\frac{1}{2}$	0	1	0	—	—	1	—	—	—	—	0	0
	10	2	2	1	—	—	0	—	—	—	—	0	0
	$10\frac{1}{2}$	3	0	0	—	—	1	—	—	—	—	0	0
	11	1	1	0	—	—	0	—	—	—	—	—	0
Outgoing tide	$11\frac{1}{2}$	0	0	0	—	—	0	—	—	—	—	—	0
	12	0	0	0	—	—	—	—	—	—	—	—	0
Low water													

*Note.*—Although no lactose fermenters were found in any quantity of 10 c.c. of Shipstall water examined, yet acid and gas were produced in 100 c.c. tubes. No change was however found in 75 c.c. tubes.

that we found during our routine examination of the plates and tubes. In all, 27 lactose fermenting organisms were subcultured. Their morphology was considered and they were examined regarding their behaviour to Gram's stain. They were tested also for motility. Their fermentation reactions were examined in peptone water containing lactose, saccharose, glucose and dulcite. Their Indole Reactions and their Vosges-Proskauer Reactions were deterred. It will be seen from Table III that the majority of these organisms proved to be "coliform" in nature.

Table III.

*Lactose fermenting organisms in sea-water.*

	Morphology	Glucose	Lactose	Saccharose	Dulcite	Indole	Litmus milk	Motility
1.	Gram-bacillus	A & G	A & G	A & G	A & G	+	A & C	+
2.	"	A & G	A & G	-	A & G	+	A & C	-
3.	"	A & G	A & G	-	-	-	A	?
4.	"	A & G	A & G	A & G	A & G	+	A & C	-
5.	"	A & G	A & G	A & G	A & G	+	A & C	+
6.	Gram+coccus	-	A & G	-	-	-	A	-
7.	Gram-bacillus	A & G	A & G	A & G	-	+	A & C	-
8.	"	A & G	A & G	A & G	A & G	+	A & C	-
9.	"	A & G	A & G	A & G	A & G	-	A & C	+
10.	"	A & G	A & G	A & G	-	+	A & C	+
11.	"	A & G	A & G	A & G	A & G	+	A & C	+
12.	"	A & G	A & G	-	A & G	+	A	-
13.	"	A & G	A & G	0	A & G	+	0	+
14.	"	A & G	A & G	A & G	A & G	+	0	-
15.	"	A & G	A & G	-	A & G	+	0	-
16.	"	A & G	A & G	0	A & G	-	0	?
17.	"	A & G	A & G	A & G	A & G	-	0	+
18.	"	A & G	A & G	-	A & G	+	0	-
19.	Gram-bacillus	A & G	A & G	0	A	-	0	-
20.	Gram+coccus	A	A	0	A	0	0	-
21.	Gram-bacillus	A	A	-	A	-	0	-
22.	"	A & G	A & G	A & G	A & G	-	0	+
23.	"	A & G	A & G	-	A	-	0	+
24.	"	A	A & G	0	A & G	+	0	-
25.	"	A	A	-	A	-	0	+
26.	Gram+coccus	A	A & G	-	A	-	0	-
27.	Gram-bacillus	A & G	A & G	-	A & G	-	0	-

*Note.*—A & G=acid and gas. +=Indole production. -=no change. 0=not examined. A=acid only. A & C=acid and clot. Nos. 1-12 are organisms obtained from sea-water in 1914. Nos. 13-27 are organisms recovered from sea-water and oysters in 1919. None of the organisms except No. 3 gave a positive Vosges-Proskauer Reaction.

It has been shown by other workers that intestinal organisms die rapidly in sea-water, and that a period of four or five days is sufficient even for a heavily infected sea-water to become practically sterilised.

The presence, therefore, of these lactose fermenters of the faecal or "coli-form" type showed that the pollution was recent. Not much interest attaches to the non-lactose fermenters which we isolated and subcultured. As is usual in enquiries of this nature, no *B.typhosus* or para-typhoid organisms were isolated.



## A SUITABLE RELAYING PLACE.

It seemed to us hardly likely that we should find a place anywhere within the harbour where the water was comparatively clean and suitable for the relaying and cleansing of oysters. If such a place was to be found it must be somewhere as far as possible from the harbour entrance; some place where the tides were not very swift, so that sedimentation and purification might have had time to take place before the water arrived at this hypothetical relaying ground. We thought that Redhorn or the top of the Wareham Channel might possibly be fairly clean, but they proved to be otherwise. Samples taken off Ower Farm and off Arne near Shipstall Point were however clean or nearly so—*B. coli* being present in 100 c.c. tubes but not in 75 c.c. tubes and five 10 c.c. plates being free from lactose fermenters (Table II). Owing to the difficulties of navigation, Ower is not a very accessible place, so we decided to relay some oysters off Shipstall Point near Arne. This place is situated at the top of the Wych Channel about three miles from the open sea. Between Arne and the sea lies Branksea Island and the water coming up to Arne takes a winding course around this: in addition, between Arne and the sea there are many acres of mud flats covered by rank weed and grass, which are awash with water when the tide is high: these practically form a filter for the sea-water. They slow the rate of the tide and must necessarily act as a mechanical filter to some of the water passing from the sea towards Arne.

Again, as this spot is so far from the open sea it is improbable that the water which forms any one high tide was actually in the open sea during the low water preceding that high tide—in other words if a single gallon of sewage contaminated water from the neighbourhood of Old Harry could be followed into Poole Harbour it would take several tides to find its way up to Shipstall Point. Indeed the actual movement of the water in these upper reaches is only slight, and this has been demonstrated by tide-floats. Something almost comparable to “storage” takes place in these places that are more remote from the sea, and self purification of the water is the natural outcome of this.

These natural conditions no doubt explain the fact that off Shipstall Point near Arne the sea-water was reasonably free from lactose fermenting organisms, and gave us a hope that the place might be suitable for the relaying of oysters. Twelve oysters were therefore dredged from the main channel. Six of them were brought straightway to the Laboratory and the remaining six were relaid in a buoyed net off Arne. The first half dozen were polluted, but not heavily; the half dozen which were relaid at Arne for seven days were clean with an average of only two lactose fermenting organisms per oyster. In view of this we decided not to pursue at present the intended part of our research which dealt with sterilisation by means of chlorine; but rather to accumulate as many facts as possible regarding the relaying at Arne.

The experiment was therefore repeated on several occasions, and always

with the same results—namely that the oysters before relaying were dirty and after relaying were clean—sometimes almost sterile (Table IV).

The greatest care was taken in the minute examination of these relaid oysters and a large number of plates of varying quantities were made. On one occasion for instance, 16 plates each of 2 c.c. were made from one oyster—practically one-third of the oyster was plated in small quantities—and only 13 lactose fermenters were found, corresponding to a total of 40 per oyster. On another occasion 0.9 part of an oyster was plated, giving a count of 12 lactose fermenters per oyster. On another occasion 25 c.c. of the emulsion were tubed in quantities of 1 c.c. in each of 25 tubes: acid and gas and a coliform organism were present in one tube—a total of four per oyster. In all 42 oysters after relaying were examined. The highest count observed was

Table IV.

*Results of relaying oysters at Shipstall.*

Before relaying		After relaying	
Date	Lactose fermenters per oyster	Date	Lactose fermenters per oyster
22. 9. 19	100	30. 9. 19	2
8. 10. 19	1600	18. 10. 19	14
8. 10. 19	1200	18. 10. 19	10
23. 10. 19	400	30. 10. 19	40
—	—	4. 11. 19	10 (a)
4. 11. 19	400	12. 11. 19	15 (b)
4. 11. 19	700	12. 11. 19	40
12. 11. 19	2580	20. 11. 19	17 (c)
12. 11. 19	1050	20. 11. 19	12 (c)
22. 11. 19	590	2. 12. 19	3 (d)

(a) These oysters were found off Arne. They had not been laid there by us.

(b) 5 oysters mixed. Quantities equal to one-third of an oyster plated.

(c) 5 oysters mixed. Quantities equal to nine-tenths of an oyster plated.

(d) 5 oysters mixed. Quantities equal to one-half of an oyster plated.

40 *B. coli* per oyster: the lowest was 2 per oyster. The average was 16 per oyster. As the result of these findings we have recommended that Shipstall Point off Arne is a safe place for the relaying of oysters; and have been able to give an assurance that oysters which have been relaid there for a week will be of reasonable bacteriological purity.

## CONCLUSION.

1. The contamination of the water in Poole Harbour comes in with the flood tide from the open sea, and does not originate within the Harbour.

2. The nearer the sea and the more rapid the current, the greater is the pollution of the Harbour water.

3. The large oyster beds near the harbour entrance and in the main channel are polluted, and the oysters in them contain many organisms derived from sewage.

4. These oysters can be cleansed by relaying off Shipstall Point near Arne where the water is comparatively free from sewage organisms.



## INDEX OF AUTHORS.

	PAGE
ARKWRIGHT, J. A., BACOT, A., and DUNCAN, F. M. The Association of <i>Rickettsia</i> with Trench Fever. (With Plates II and III) . . . . .	76
BACOT, A., <i>see</i> ARKWRIGHT	
BRADLEY, B., <i>see</i> CLELAND	
BROWNING, C. H. and GULBRANSEN, R. The Testing of Antiseptics in Relation to their Use in Wound Treatment . . . . .	33
CAMPBELL, A. W., <i>see</i> CLELAND	
CLELAND, J. B. and BRADLEY, B. Further Experiments in the Etiology of Dengue Fever. (Clinical notes by MACDONALD, W.) (With 9 Charts) . . . . .	217
CLELAND, J. B. and CAMPBELL, A. W. An Experimental Investigation of an Australian Epidemic of Acute Encephalo-mylitis . . . . .	272
CRAMER, W. and GYE, W. E. A Note on "Defence Rupture" and the Action of Electrolytes . . . . .	463
DUDGEON, LEONARD S. On the Effects of Injections of Quinine into the Tissues of Man and Animals. (With Plate III) . . . . .	317
DUNCAN, F. M., <i>see</i> ARKWRIGHT	
EAGLETON, A. J. The Bacterial Content of the Air in Army Sleeping Huts, with special reference to the Meningococcus. (With 1 Diagram) . . . . .	264
EWART, R. J. The Influence of the Age of Parent at Birth of Offspring upon the Development of Eye Colour and Intelligence—A Correction . . . . .	95
FAIRLEY, N. HAMILTON. The Laboratory Diagnosis of Typhus Fever. Further Observations on the value and on the significance of the Weil-Felix Reaction . . . . .	203
FERGUSON, MARGARET. The Diets of Labouring Class Families during the course of the War . . . . .	409
GRAHAM-SMITH, G. S. Some Factors influencing the Actions of Dyes and Allied Compounds on Bacteria. (With Plate I and 6 Charts) . . . . .	1
GRIFFITH, A. STANLEY. The Cultivation of <i>Spirochaeta icterohaemorrhagiae</i> and the Production of a Therapeutic Anti-spirochaetal Serum . . . . .	59
GULBRANSEN, R., <i>see</i> BROWNING	
GYE, W. E., <i>see</i> CRAMER	
HORT, EDWARD C. The Cultivation of Aerobic Bacteria from Single Cells. (With 1 Text-figure) . . . . .	361
HORT, EDWARD C. The Reproduction of Aerobic Bacteria. (With Plates IV—VII) . . . . .	369
KAUNTZE, W. H. A Polyvalent Vaccine in the Treatment of Bacillary Dysentery in East Africa . . . . .	417
KENNAWAY, E. L. and WRIGHT, A. M. Two Instances of Human Sera showing Abnormal Anti-complementary Power . . . . .	255
MACDONALD, W., <i>see</i> CLELAND	
MACKIE, T. J. The Atypical Dysentery Bacilli . . . . .	69
MCLEOD, J. W. Observations on the Cultivation of Typhoid and Paratyphoid Bacilli from the Stools with special reference to the Brilliant Green Enrichment Method . . . . .	260
NANKIVELL, A. T. and STANLEY, J. M. The Contamination of Oysters. (With 1 Chart) . . . . .	465
SAVAGE, WILLIAM G. Cats and Human Diphtheria . . . . .	448
SHEARER, C. Studies on the Action of Electrolytes on Bacteria. Part I. The Action of Monovalent and Divalent Salts on the Conductivity of Bacterial Emulsions. (With 8 Charts) . . . . .	337
STANLEY, J. M., <i>see</i> NANKIVELL	
TULLOCH, W. J. Report of Bacteriological Investigation of Tetanus carried out on behalf of the War Office Committee for the Study of Tetanus. (With 7 Diagrams) . . . . .	103
WOOD, DENYS R. Recent Advances in the Differentiation of Lactose-fermenting (Gas-producing) Bacilli, with special reference to the Examination of Water and Food Products . . . . .	46
WRIGHT, A. M., <i>see</i> KENNAWAY	

## INDEX OF SUBJECTS

	PAGE
Acriflavine, <i>see</i> Antiseptics	
Age of parent, influence on eye-colour and intelligence of offspring . . . . .	95
Air, <i>see</i> <i>Meningococcus</i>	
Antiseptics, testing of, in relation to wounds . . . . .	1, 33
Bacilli, <i>see</i> Bacteria	
<i>Bacillus</i> , <i>see</i> Bacteria	
BACTERIA, action of dyes on . . . . .	1, 33, 44
action of electrolytes on . . . . .	337, 463
agglutinability, effect of reproductive life of bacteria on their . . . . .	382
cultivation of, from single cells . . . . .	361
involution forms of . . . . .	370
plasmoptysis and plasmolysis of . . . . .	372
reproduction of . . . . .	369
<i>see</i> Wound infection	
Bacilli, lactose fermenting, in water and food . . . . .	47
<i>Bacillus anthracis</i> , action of electrolytes on . . . . .	350 <i>et seq.</i>
<i>cloacae</i> in polluted water . . . . .	48
<i>coli</i> , action of dyes on . . . . .	1, 35
action of electrolytes on . . . . .	342 <i>et seq.</i>
in oysters . . . . .	465
in polluted water . . . . .	49 <i>et seq.</i>
<i>diphtheriae</i> , <i>see</i> Diphtheria	
<i>dysenteriae</i> , agglutinability affected by its reproductive life . . . . .	387 <i>et seq.</i>
atypical . . . . .	69
<i>lactis aerogenes</i> in polluted water . . . . .	48
<i>paratyphosus</i> , cultivation from stools, brilliant green method . . . . .	260
<i>proteus</i> in relation to typhus . . . . .	205
<i>pyocyaneus</i> , action of dyes on . . . . .	1
<i>tetani</i> , investigations on, <i>see</i> special index, p. 103	
<i>typhosus</i> , agglutinability affected by its reproductive life . . . . .	384 <i>et seq.</i>
cultivation of, from single cells . . . . .	366 <i>et seq.</i>
cultivation of, from stools, brilliant green method . . . . .	260
reproduction of . . . . .	373 <i>et seq.</i>
<i>typhi-exanthematicus</i> Plotz in relation to typhus . . . . .	205
<i>welchii</i> , effect of electrolytes on . . . . .	464
in oysters . . . . .	465
in wounds . . . . .	136 <i>et seq.</i> , 188
<i>Meningococcus</i> . . . . .	337, 346
action of electrolytes on . . . . .	342 <i>et seq.</i>
agglutinability affected by its reproductive life . . . . .	390
in air of Army Sleeping huts, tests of presence . . . . .	264
<i>Pneumococcus</i> , action of electrolytes on . . . . .	356
<i>Staphylococcus aureus</i> , action of dyes on . . . . .	1, 37
<i>Streptococci</i> in oysters . . . . .	465
in polluted water . . . . .	49 <i>et seq.</i>
<i>Vibrion septique</i> , effect of electrolytes on . . . . .	463
in wounds . . . . .	136 <i>et seq.</i>



	PAGE
Cats, <i>see</i> Diphtheria	
Cerebro-spinal fever, <i>see</i> <i>Meningococcus</i>	
Crystal violet, <i>see</i> Antiseptics	
<i>Culex fatigans</i> , in relation to Dengue fever . . . . .	217, 232
Dengue fever, experiments on etiology of . . . . .	217 <i>et seq.</i>
by blood injection in man . . . . .	219, 234
by infected mosquitoes . . . . .	232
on animals . . . . .	233
Dengue fever, nature of virus of . . . . .	222 <i>et seq.</i>
pyrexia and pulse, rashes, symptoms, etc. . . . .	220 <i>et seq.</i>
Diets of labouring class families during war . . . . .	409
Diphtheria, in relation to Cats and Man . . . . .	448
Dyes, <i>see</i> Bacteria	
Dysentery, bacillary, in East Africa, polyvalent vaccine treatment of . . . . .	417 <i>et seq.</i>
pathology of . . . . .	420
symptoms, physical signs, complications of . . . . .	422 <i>et seq.</i>
treatment of, diet in . . . . .	425 <i>et seq.</i>
<i>see</i> <i>Bacillus</i>	
Electrolytes and "defence rupture," <i>see</i> Bacteria, action of, etc.	
Encephalo-myelitis, acute, Australian epidemic, investigation of, <i>see</i> special index . . . . .	272
Epidemic diseases, <i>see</i> Encephalo-myelitis, cerebro-spinal fever	
Eye-colour, influence of age of parent on . . . . .	95
Food, <i>see</i> Diet	
Food-products, <i>see</i> Bacilli	
Helminth infections in Dysentery patients, etc., East Africa . . . . .	419
Homoflavine, <i>see</i> Antiseptics	
Immunity, <i>see</i> Serum, Dysentery	
Intelligence, influence of age of parent on . . . . .	95
Malaria, <i>see</i> Quinine	
<i>Meningococcus</i> , <i>see</i> Bacteria	
Mercury perchloride, testing of, in relation to wounds . . . . .	33
Mosquitoes, <i>Culex fatigans</i> and <i>Stegomyia fasciata</i> , etc., in relation to Dengue fever . . . . .	217, 232
Oysters, contamination of . . . . .	465
<i>Pediculus humanus</i> , <i>Rickettsia</i> in trench fever infected- . . . . .	76 <i>et seq.</i>
typhus fever infected- . . . . .	76
Phenol, testing of, in relation to wounds . . . . .	33
<i>Pneumococcus</i> , <i>see</i> Bacteria	
Poole harbour, contamination of oysters in . . . . .	465
Proflavine, <i>see</i> Antiseptics	
Quinine, effects of injection into tissues in man and animals . . . . .	317
Quinone, <i>see</i> Antiseptics	
<i>Rickettsia</i> in <i>Pediculus humanus</i> . . . . .	76
sheep kid ( <i>Melophagus ovinus</i> ) . . . . .	76
Trench Fever . . . . .	76
typhus . . . . .	76

	PAGE
Serum, human, with abnormal anti-complementary power . . . . .	255
<i>Spirochaeta icterohaemorrhagiae</i> , cultivation and antiserum for . . . . .	59
<i>Staphylococcus</i> , <i>see</i> Bacteria	
<i>Stegomyia fasciata</i> , proved to convey Dengue fever . . . . .	217, 232
Tetanus, bacteriological investigation on behalf of War Office . . . . .	103
Trench fever, <i>Rickettsia</i> in . . . . .	76
Typhus, Laboratory diagnosis of (Weil-Felix reaction) . . . . .	203
<i>Rickettsia</i> in . . . . .	76 <i>et seq.</i>
Vaccine treatment, <i>see</i> Dysentery	
Water, <i>see</i> Bacilli	
Wound infection, part played by certain salts in, discussed . . . . .	352
treatment, testing antiseptics in relation to . . . . .	1, 33



1

# THE JOURNAL OF HYGIENE

CAMBRIDGE UNIVERSITY PRESS

C. F. CLAY, MANAGER

LONDON: FETTER LANE, E.C. 4



H. K. LEWIS & CO., LTD., 136, GOWER STREET, LONDON, W.C. 1  
WILLIAM WESLEY & SON, 28, ESSEX STREET, STRAND, LONDON, W.C. 2

CHICAGO: THE UNIVERSITY OF CHICAGO PRESS  
(Agent for the United States and Canada)

BOMBAY, CALCUTTA, MADRAS: MACMILLAN & CO., LTD.

TOKYO: THE MARUZEN-KABUSHIKI-KAISHA

*All rights reserved*



# THE JOURNAL OF HYGIENE

EDITED BY

GEORGE H. F. NUTTALL, M.D., PH.D., SC.D., F.R.S.

QUICK PROFESSOR OF BIOLOGY IN THE UNIVERSITY OF CAMBRIDGE

IN CONJUNCTION WITH

JOHN S. HALDANE, M.D., LL.D., F.R.S.

LATE READER IN PHYSIOLOGY IN THE UNIVERSITY OF OXFORD

CHARLES J. MARTIN, C.M.G., M.B., D.Sc., F.R.S.

DIRECTOR OF THE LISTER INSTITUTE, LONDON

J. C. G. LEDINGHAM, M.B., D.Sc.

BACTERIOLOGIST-IN-CHIEF, LISTER INSTITUTE, LONDON

G. S. GRAHAM-SMITH, M.D., F.R.S.

UNIVERSITY LECTURER IN HYGIENE, CAMBRIDGE

MAJOR GREENWOOD, M.R.C.P. (LOND.)

MEDICAL OFFICER (MEDICAL STATISTICS), MINISTRY OF HEALTH

VOLUME XIX. 1920-21

CAMBRIDGE  
AT THE UNIVERSITY PRESS

1921





# CONTENTS

## No. 1 (JULY)

	PAGE
BRUCE, SIR DAVID. Tetanus. Analysis of 1458 Cases, which occurred in Home Military Hospitals during the years 1914—1918. (With 12 Diagrams and 1 Curve) . . . . .	1
GRIFFITH, A. STANLEY. A Study of the Serological Reactions of Meningococci and an account of the Method of Preparation of Anti-meningococcus Serum. (With 2 Charts) . . . . .	33
BUXTON, P. A. Carriage of Coliform Bacilli by the Oriental Hornet ( <i>Vespa orientalis</i> Fabr.) . . . . .	68
SHEARER, C. On Defense Rupture and the Antagonistic Action of Salts .	72
GREEN, IONE H. Report of Experiments on the Cold Storage of Herrings carried out at North Shields . . . . .	75
CUMMING, R. H. and MATTICK, A. T. R. An Enquiry concerning the State of Cleanliness of Empty Milk Churns . . . . .	84
BROWNING, C. H. and KENNAWAY, E. L. Suggestions for a New Criterion of a Positive Wassermann Reaction based on an Analysis of 2334 Quantitative Tests. (With 3 Text-figures) . . . . .	87
CUNNINGHAM, ANDREW and THORPE, B. A. The Milk Supply of the City of Edinburgh . . . . .	107
WILSON, W. JAMES. The Wilson-Weil-Felix Reaction in Typhus Fever .	115
Notice: The Second International Congress of Comparative Pathology .	131

## No. 2 (OCTOBER)

GRAHAM-SMITH, G. S. The Behaviour of Bacteria in Fluid Cultures as indicated by Daily Estimates of the Numbers of Living Organisms. (With 29 Charts and 1 Text-figure) . . . . .	133
FREDERICK, ROBERT C. The Conversion of Air into a Lethal Mixture of Gases by Storage of Tobacco and other Vegetable Substances . .	205
DUDGEON, LEONARD S. Blackwater Fever . . . . .	208

## No. 3 (JANUARY)

	PAGE
GRABHAM, GEORGE WALTER. Some factors in Thermal Sanitation in the Tropics. (With Plate I, 3 Charts and 3 Text-figures) . . . . .	245
HEHEWERTH, F. H. and KOP, W. A. The Wassermann Test in Patients affected with Malaria in the Tropics . . . . .	277
BLOCH, C. E. Clinical investigation of Xerophthalmia and Dystrophy in Infants and Young Children ( <i>Xerophthalmia et Dystrophia Alipogenetica</i> ) (With Plates II—IV and 5 Charts) . . . . .	283
MCDOWALL, R. J. S. The Water Supply of the Egyptian Expeditionary Force, with Special Reference to the Efficiency of Mechanical Rapid Filtration with Chlorination . . . . .	305
MALTANER, FRANK and HOPPE, ELLA. The Chemotactic Effect of Osmosis upon Leucocytes . . . . .	309
GILL, C. A. Malaria in England, with Special Reference to the Rôle of Temperature and Humidity . . . . .	320

## No. 4 (MARCH)

GARDNER, A. D. A Valuation of the "Agglutinability-factor" in Dreyer's System . . . . .	333
PRATT-JOHNSON, J. The Distribution of Malaria in South Africa and a Mosquito Survey of Military Hospital Areas . . . . .	344
TOPLEY, W. W. C. The Spread of Bacterial Infection. Some Characteristics of Long-continued Epidemics. (With 5 Charts) . . . . .	350
LLOYD, D. JORDAN, CLARK, A. B. and MCCREA, E. D. On Rope (and Sourness) in Bread. Together with a Method of estimating Heat-resistant Spores in Flour . . . . .	380
DURHAM, HERBERT E. The Prevalence of Thyroid Enlargement in and about Hereford . . . . .	394
INDEX OF AUTHORS . . . . .	402
INDEX OF SUBJECTS . . . . .	404



TETANUS.

ANALYSIS OF 1458 CASES, WHICH OCCURRED IN HOME  
MILITARY HOSPITALS DURING THE YEARS 1914—1918

BY SIR DAVID BRUCE, K.C.B., F.R.S.,

Major-General, Army Medical Service.

Chairman, War Office Committee for the Study of Tetanus.

(With 12 Diagrams and 1 Curve.)

CONTENTS.

	PAGE
Introduction . . . . .	1
I. The Incidence of Tetanus among the wounded . . . . .	4
II. The Distribution of cases of Tetanus by months . . . . .	6
(a) From date of wound . . . . .	6
(b) From date of onset . . . . .	6
III. Geographical Distribution . . . . .	6
IV. Classification of various types of Tetanus . . . . .	9
(a) Four types or varieties . . . . .	9
(b) General and local . . . . .	10
V. Period of Incubation . . . . .	10
(a) At daily intervals . . . . .	10
(b) At weekly intervals . . . . .	10
(c) Relation of incubation to rate of mortality . . . . .	12
(d) Variation in period of incubation . . . . .	12
VI. Rate of Mortality . . . . .	14
(a) Gross rate of mortality . . . . .	14
(b) At different periods of the war . . . . .	15
(c) In relation to position of wounds . . . . .	15
(d) The effect of fractures on rate of mortality . . . . .	16
(e) After operation on site of wound . . . . .	16
(f) Operation after onset of tetanus . . . . .	17
(g) In relation to period of incubation . . . . .	17
VII. Prophylactic Treatment of Tetanus . . . . .	18
(a) Incidence among protected and unprotected . . . . .	19
(b) Incubation period in protected and unprotected . . . . .	20
(c) Rate of mortality in protected and unprotected . . . . .	20
(d) Effect of multiple inoculations . . . . .	21
(e) Effect of multiple inoculations on period of incubation . . . . .	21
(f) Effect of multiple inoculations on rate of mortality . . . . .	22
(g) Effect of time of inoculation on rate of mortality . . . . .	23
(h) Increase of dose to 1500 units . . . . .	23
(i) The addition to serum of other antitoxins . . . . .	24
VIII. Therapeutic Treatment of Tetanus . . . . .	24
(a) The best route . . . . .	26
(b) The best dosage . . . . .	27
(i) The total quantity given . . . . .	28
(ii) The average daily quantity . . . . .	29
IX. Types of Tetanus Bacilli . . . . .	30
X. Anaphylaxis . . . . .	30

## INTRODUCTION.

THE purpose of this paper is to place on record the available figures relating to cases of tetanus which occurred in Home Military Hospitals during the Great War of 1914–1918. It may be considered that some of the tables give no very useful or practical information, but they are nevertheless recorded in order that medical officers at the outbreak of some future war may have the opportunity of learning what was done for the prevention and treatment of tetanus during this war. For example nothing can be gained from a study of the figures given in this analysis with regard to the curative or therapeutic value of antitetanic serum, but it may be of some antiquarian interest half a century hence to know the amount given and the mode of administration in the years 1914–1918.

The total number of British wounded in all the theatres of war has been officially reported as 2,032,142. If the number 2385 be taken as the total number of cases of tetanus which occurred among these wounded then the incidence of cases of tetanus to wounded is 1·17 per 1000.

The total number of wounded treated in Home Hospitals has been calculated to be about 1,242,000. The number of cases of tetanus arising among them was 1458, giving an incidence of 1·2 per 1000.

It is the analysis of these 1458 cases which forms the subject of this paper. It must then be distinctly understood that only cases of tetanus arising in England are dealt with. Cases occurred in France and were treated in Military Hospitals in France, among wounded soldiers, before they had an opportunity of being transferred to England. These have been analysed at least in part by Sir William Leishman and Major Smallman, and later by Colonel Cummins and Major Gibson.

Tetanus made its appearance very shortly after hostilities had commenced and it soon became evident that vigorous steps would have to be taken to save our men from this painful and very fatal disease. It had been expected, of course, that there would be a few cases of tetanus among the wounded men and provision had been made to deal with such cases when they arose. Nevertheless when it became possible to obtain figures showing the incidence of tetanus it was seen that the numbers were considerably higher than had been anticipated. Lord Kitchener, then Secretary of State for War, and the Director-General, Army Medical Services, accordingly determined to lose no time in grappling with the danger.

My connexion with this work was due to an order from the then Director-General, Sir Alfred Keogh, G.C.B., who wrote in October 1914, "It is hoped that you will direct your attention specially to the subject of Tetanus, with a view to elucidating the problems of causation and cure." Hence the taking up of this kind of work, which was quite new to me, was due to a request from the Director-General and not from any volition on my own part.



In March 1916, a Tetanus Committee was formed at the suggestion of Prof. Waller, F.R.S., the late Prof. Plimmer, F.R.S., and Captain Golla, R.A.M.C. The first meeting took place on the 7th March of that year. A Memorandum on Tetanus, which is now in its fourth edition, was prepared by the committee and circulated; the Tetanus Report forms were revised, and a special form prepared for the use of the inspectors of Tetanus. Copies of these documents are attached, as they may be found of some use in the future.

There were some forty Inspectors of Tetanus stationed in different parts of the country whose duty it was to visit cases of tetanus as soon as they occurred, and to advise or assist when necessary in the treatment. These inspectors were usually expert clinicians, neurologists or bacteriologists, and were of the greatest service in establishing sound methods in the nursing and treatment of the cases.

They sent in an independent report which was useful to compare with the report sent in afterwards by the Medical Officer in charge of the case. The inspectors were without exception all of them keen on the work, and I consider the idea of detailing officers for this purpose in every important centre in the three kingdoms to have been the most useful and most practical part in the organisation for the study and care of the cases of tetanus.

Members of the Tetanus Committee have written several papers in the Medical Journals with a view to giving medical officers the latest developments in our knowledge of tetanus. A list of these papers is given in an appendix. The Committee also instituted various original investigations. Capt. Golla, R.A.M.C., worked at different problems at the Physiological laboratory of the University of London, Capt. Tulloch, R.A.M.C., at the Lister Institute, and Prof. Sherrington in his own laboratory at Oxford. The expenses of these various researches, amounting to £1350, were borne by the Lister Institute, without which generous aid the Committee would have been unable to carry out any of this research work.

In conclusion, my best thanks are due to Lady Bruce for the immense amount of work she has undertaken in the preparation of this Analysis. To her fell the irksome task of collecting and arranging under their different heads, the various particulars contained in the Inspectors' Reports and the Tetanus Returns.

This has been an undertaking of a particularly arduous and tiresome character demanding qualities of patience and industry especially when dealing with Reports often written with extreme haste and illegibility.

I would also wish to express my best thanks to all the Inspectors of Tetanus who were, in the main, responsible for carrying out the Committee's recommendations; to the Officers Commanding Hospitals, Medical Officers in charge of cases, and to the nursing Sisters to whom falls the heaviest burden in the care of the cases.

# 1. THE INCIDENCE OF CASES OF TETANUS AMONG THE WOUNDED TREATED IN HOME MILITARY HOSPITALS.

In regard to the incidence of tetanus among the wounded soldiers treated in home military hospitals, it is impossible to deal at all fully. The number of wounded sent over to England from the Western Front between August 1914 and November 1918 was roughly 1,242,000. The number of cases of tetanus which occurred during this period in home hospitals was 1458. This gives a rate of incidence of 1·2 per 1000. It must be confessed that these figures can only be approximate. It will probably be years before the correct figures are available. For example many cases of "gassing" were included among the wounded. For the purpose of adjudicating pensions this may be useful but for purposes of tetanus statistics it introduces a fallacy.

**Diagram 1** is an attempt to give the ratio of the number of cases of tetanus per thousand of the wounded landed in England during each month, from August 1914 to the close of the war on November 11th, 1918.

The cases of tetanus are reckoned from the date of wound not from the date of onset. For example, 2161 wounded men arrived in England from overseas during August 1914. Eight of these men were at some time attacked by tetanus, giving a ratio of 3·7 per 1000.

From this diagram it will be seen that the ratio of the number of cases of tetanus occurring in home military hospitals to the number of wounded sent over to England is 3·7 per 1000 in August 1914; 9 per 1000 in September, and that it rapidly drops to 1·4 in December, at which lower rate it continues practically during 1915. There is a tendency to rise during 1916 due it may be to an improvement in the diagnosis and subsequent increase of cases of local tetanus, which at the beginning of the war were possibly overlooked. This would naturally raise the ratio of the incidence.

It will also be seen that the incidence of the disease tends to become lower as the war goes on. It is probable that this is due after the middle of 1917 to more thorough surgical treatment. It was stated in the second analysis of cases of tetanus in 1915 that "if thorough surgical treatment is carried out on wounds from the beginning so as not to allow the presence of necrotic tissues or foreign bodies, the number of cases of tetanus should sensibly diminish if not altogether disappear." The surgeons took a long time to learn how to do this, but in the introduction of primary excision and primary or delayed primary suture the most remarkable results were obtained, and if it had not been for the sudden cessation of the war, it is probable that tetanus would have become almost extinct among the British wounded. The most interesting feature in the diagram is the sudden drop in 1914 from 9 per 1000 in September to 1·4 per 1000 in December. This was undoubtedly due to the introduction of prophylactic injections of antitetanic serum which did not come into force until about the middle of October. This topic will be discussed more fully when the prophylactic or preventive treatment of tetanus comes under review.



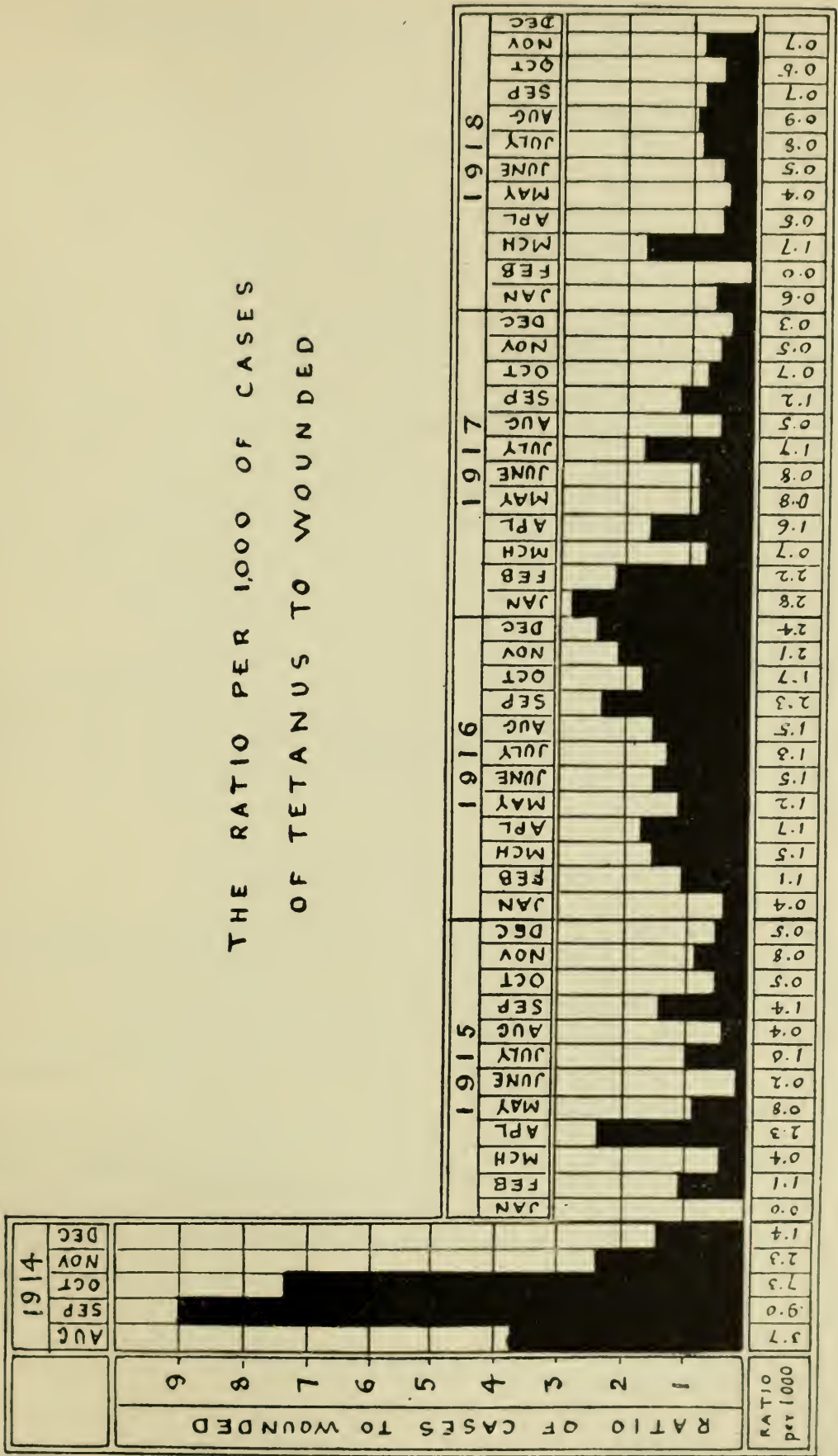


Diagram 1.

## II. THE DISTRIBUTION OF CASES OF TETANUS FROM AUGUST 1914—SEPTEMBER 1918.

**Diagram 2** merely represents the number of cases of tetanus which have been treated in home military hospitals since the beginning of the war. They are taken from the date of wound, not from the date of onset of the disease. The figures have no relation to the number of wounded or the number of troops engaged. The diagram merely shows periods of activity and inactivity in the fighting line. For example the eight men in August 1914 were wounded at the battle of Mons, the 54 in September during the fighting at the Marne and at the Aisne, and the 121 in October and November at the first battle of Ypres when we had some 13,000 wounded. The rise in April and May 1915, marks the second battle of Ypres and Festubert, 60,000 wounded, and in July and September 1916 the battle of the Somme which caused some 300,000 casualties before the operations were over. The rise in April 1917 marks the battle of Vimy and Arras; March 1918 the great German offensive, and in August, September and October, our final attack on the German lines which led up to the Armistice of 11th November 1918. The casualties during the last hundred days amounted to 300,000.

**Diagram 3** represents the number of cases of tetanus which have been treated in home military hospitals since the beginning of the war. They are taken from the day of onset of disease, not from date of wound. For example, in August 1914 there were three wounded men in England who had been attacked by tetanus.

## III. GEOGRAPHICAL DISTRIBUTION.

It may be possible in the future when fuller figures are collected to differentiate between different districts. In one district the occurrence of tetanus may have been much more common than in another.

Bullock and Cramer have shown that the presence of calcium salts in the earth may have a determining effect on the occurrence or non-occurrence of tetanus and gas gangrene.

It is well-known that tetanus bacilli or their spores, if freed from toxin, are incapable of giving rise to the disease when injected into healthy animals. The same is true of the bacilli of gas gangrene. Bullock and Cramer set themselves to discover the missing factor in the etiology of these diseases and state that they found it in injections of small doses of a soluble ionisable calcium salt. They state that these observations account satisfactorily for the curious fact that the occurrence of gas gangrene on the Western Front was very "patchy."

Bullock does not think that the naturally occurring rocks are of great importance; it is rather the manures spread on the land which supply the calcium salts which set in motion the tetanus and gas gangrene bacilli.



CASES OF TETANUS BY MONTHS FROM DATE OF WOUND.

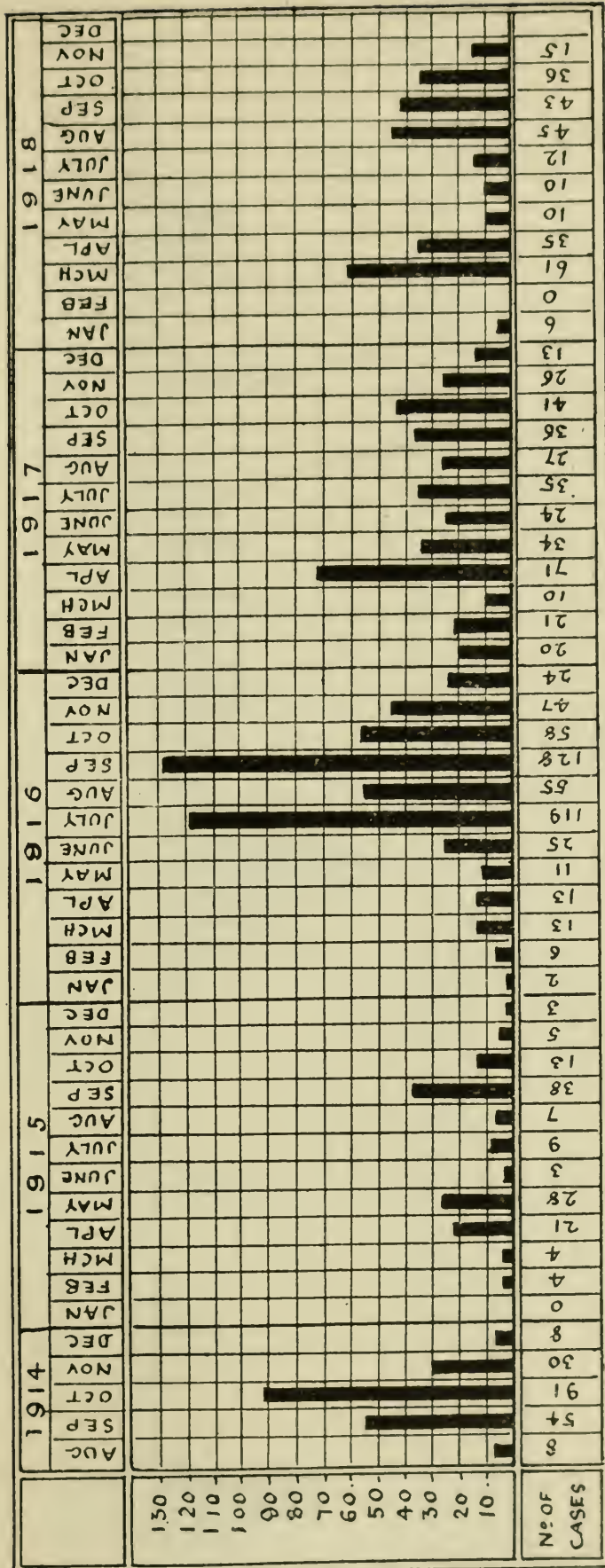


Diagram 2. Number of cases of Tetanus occurring in England during the war from date of wound.

CASES OF TETANUS BY MONTHS FROM DATE OF ONSET OF DISEASE

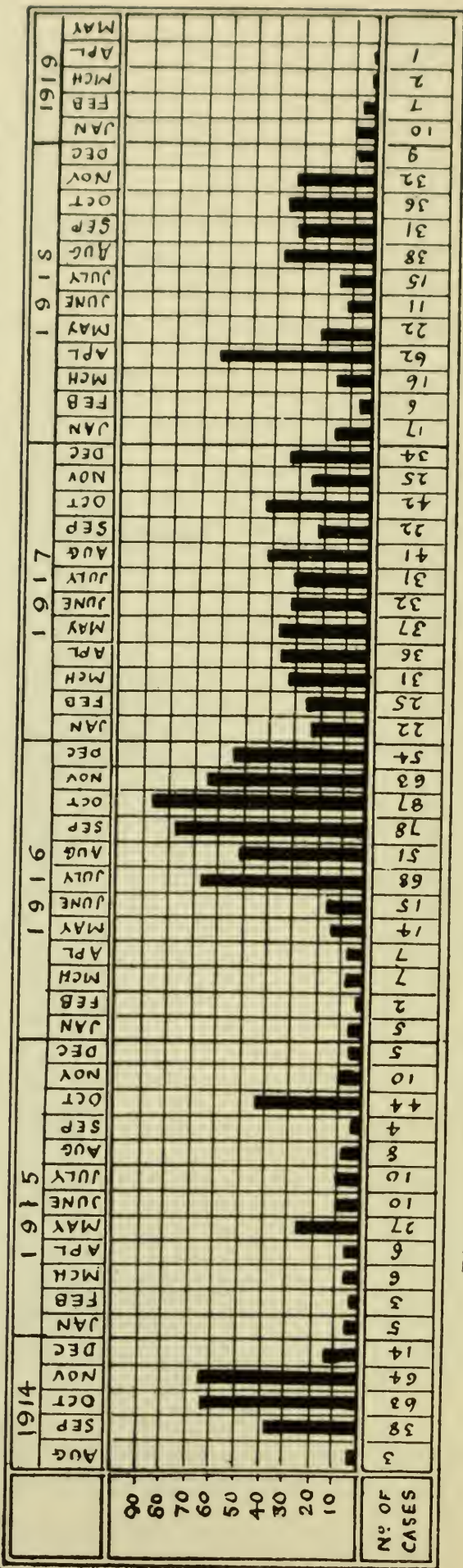


Diagram 3. Number of cases of Tetanus occurring in England during the war from date of onset.



After reading their paper an attempt was made to discover if any difference in the occurrence of cases of tetanus due to the geological formation could be made out.

To do this it is evident that the number of wounded, and the incidence of tetanus among them, occurring on a particular formation, for example the chalk, would require to be known.

This then could be compared with the incidence of tetanus among the same number of men wounded on another formation. For example, if in district A 100,000 wounded had an incidence of tetanus of 10 per 1000, and in district B the same number had only an incidence of 1 per 1000 a case would be made out. It might of course be due as Bullock thinks to district A being more intensively cultivated than the other, or it might be due to some chemical substance found in one formation and not in the other.

On consulting a geological map of the part of the Western Front held by the British it was seen that it all lay on a chalk formation covered over in patches by more recent gravels and sands. It seems therefore that it is very unlikely that any information on this subject would be gained by a comparative study of this front.

A spot-map was prepared showing as nearly as possible the position of wounded men who afterwards developed tetanus. After this was made it was evident that nothing could be gained from it. The information is not exact enough. Perhaps later on when more facts are available something might emerge. For the present it must be sufficient to draw attention to Bullock and Cramer's paper with the hope that further investigation may throw light on what promises to be a very interesting subject.

#### IV. CLASSIFICATION OF VARIOUS TYPES OF TETANUS.

The following table classifies as far as possible the cases of tetanus under four headings, as laid down in the third edition of the Memorandum on Tetanus.

Table I.

	Cases	Recovered	Died	Mortality per cent.
1. Trismus the <i>earliest</i> symptom				
(a) With complete closure of jaws developing within 24 hours after onset of symptoms	77	22	55	71.4
(b) With complete closure of jaws developing after 24 hours of onset	80	40	40	50.0
(c) With incomplete closure of jaws ... ..	505	380	125	24.9
2. Trismus occurring after other symptoms of tetanus have shown themselves ... ..	134	109	25	18.6
3. General tetanus without trismus ... ..	50	38	12	24.0
4. Local tetanus ... ..	201	201	—	0.0

Table I should be useful for prognosis. If trismus is the first symptom and is complete within 24 hours, the prognosis is bad, seven dying in ten; if the trismus occurs after other symptoms have declared themselves then only two die in ten.

As this classification has not been in use during the whole of the war, it has not been found possible to classify in this way all the 1458 cases under consideration. Many of the reports especially at the beginning of the war were sent in without any attempt at classification and at first cases of local tetanus either did not occur or were overlooked. These unclassified cases, 411 in number, were sent in simply with the diagnosis of tetanus, but as they occurred mostly at the beginning of the war and had a high rate of mortality it may be presumed that most of them were cases of general tetanus with trismus.

Table II represents the proportion of general to local tetanus as reported at different periods of the war.

Table II.

Type	Percentages				
	1914	1915	1916	1917	1918
General	98.9	98.6	87.0	76.6	83.5
Local ...	1.1	1.4	13.0	23.4	16.5

It is manifest that the ratio of cases of local tetanus to cases of general tetanus tended to become higher each year. This is due in part no doubt to the introduction of the prophylactic injection of antitoxin, also to an improvement in the diagnosis of mild and obscure cases of tetanus. What in the first years of the war would be considered to be due to a non-specific irritation of nerve and muscle, came afterwards to be recognised as a local manifestation of tetanus.

## V. THE PERIOD OF INCUBATION.

### (a) *At daily intervals.*

The incubation period, or the number of days which elapse between the date of wound, and the onset of tetanus symptoms, is represented by the following curve.

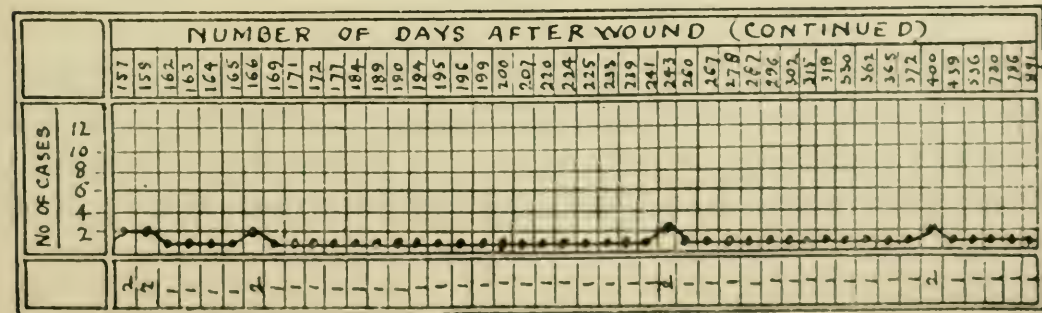
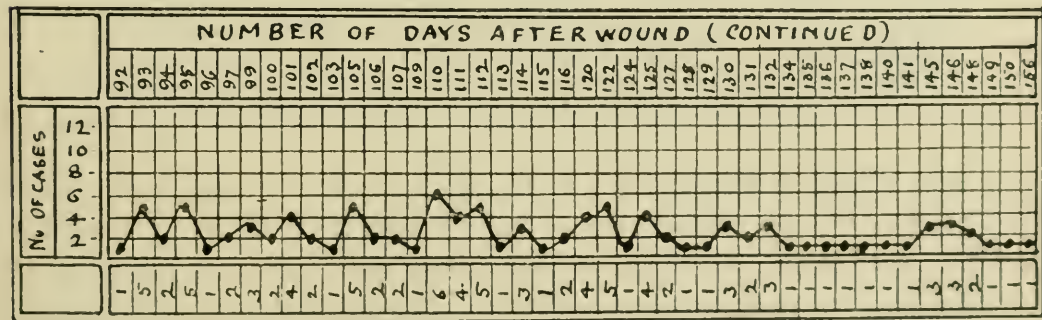
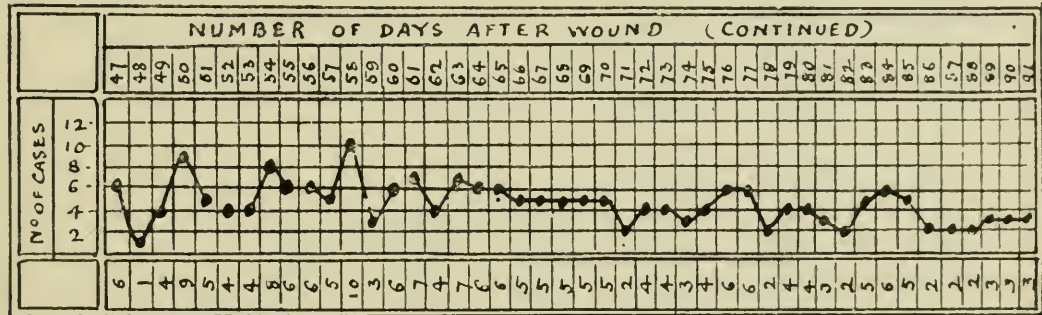
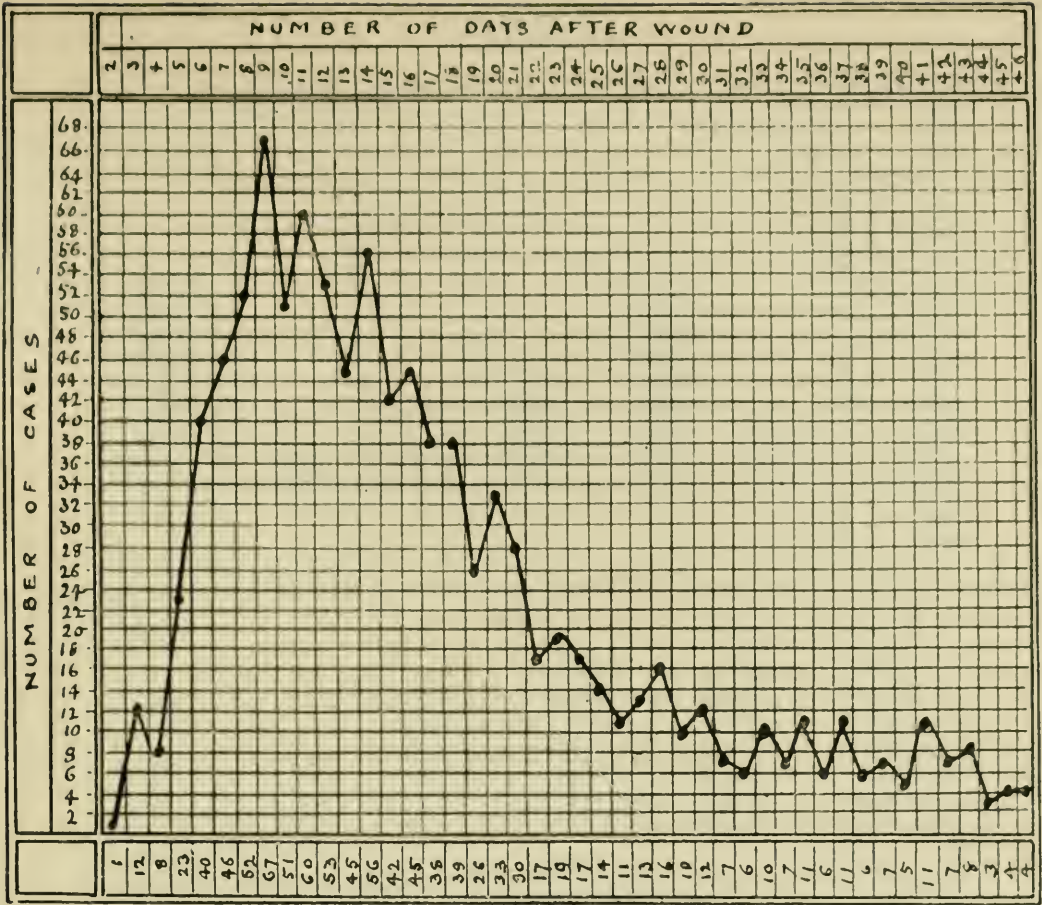
**Curve A** shows the number of cases which occurred on each day after the date of wound. The shortest period of incubation was two days, the longest two and a half years. The greatest number of cases occurred on the ninth day.

### (b) *Periods of Incubation shown at weekly intervals.*

**Diagram 4** gives the percentages of cases of tetanus occurring at weekly intervals.

From the above diagram it will be seen that in the 1458 cases under consideration, 9.5 per cent. of the cases are attacked during the first week; 28 per cent. during the second, and as many as 31.7 per cent. after five weeks. This diagram is very different from one made in pre-serum days, and as we shall afterwards see, is due to the prophylactic injection of antitoxin given soon after the wound is received.





Curve A.





as it was not until the middle of October that the administration of anti-toxin had got under weigh.

**Diagram 6** gives the average periods of incubation in days for each year of the war. For example, there were 138 cases of tetanus among the wounded treated in England in 1914; these had an average incubation period of only 11·8 days. In 1915 the average incubation period had risen to 27·3 days, in 1916 to 34 days; in 1917 to 48 days; and in 1918-19 to 50 days.

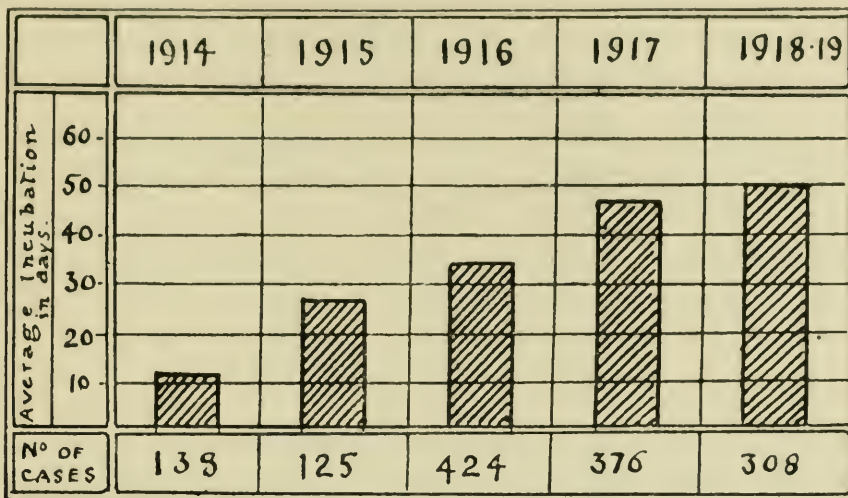


Diagram 6. The lengthening of the Period of Incubation since the beginning of the War.

In France the average period of incubation was much shorter. From July 1st to October 31st, 1916, there were 157 cases, with an average incubation of 12 days; from November 1st, 1916 to December 31st, 1917, 342 cases with an incubation of 13·2 days. This of course is due to the fact that the acute cases with a short incubation period would be treated in France; those with a longer incubation would have time to be transferred to England.

Table III gives the number, in percentages, of cases of tetanus with short, medium and long incubation periods which have occurred since the beginning of the war.

Table III.

*Short, Medium, and Long Incubation periods since the Beginning of the War.*

	Up to 10 days, per cent.	11 to 22 days, per cent.	More than 22 days, per cent.
1914	52·8	43·6	3·6
1915	29·9	40·8	29·9
1916	13·8	39·4	46·8
1917	15·2	26·8	58·0
1918	24·9	35·6	39·5

It will be seen that in 1914, 52·8 per cent. of the cases had a short incubation period of 10 days or under; this fell to 13·8 per cent. in 1916 and 15·2 per cent. in 1917. In 1918, however, it rose somewhat, about a quarter of the cases of tetanus in the last year of the war having a short incubation period. In

1914 there were only 3·6 per cent. of the cases with a long incubation period whereas this rose in 1917 to 58 per cent.

This is a remarkable result, and can be taken as a measure of the good effect of the prophylactic inoculation of antitetanic serum. During the first months of the war, as has already been pointed out, many of the wounded did not receive a primary injection, hence the number of acute cases with a short incubation. This introduction of the prophylactic inoculation of antitetanic serum in war wounds has changed the whole picture of tetanus, from being an acute disease almost invariably fatal to a chronic disease with a case mortality of only some 20 per cent.

The rise in 1918 is probably due to the rapid movement of troops and the large number of wounded to be dealt with. On account of this it is justifiable to believe that many of the wounded men did not receive their primary prophylactic injection of antitetanic serum, hence the lowering of the average incubation period in the last year of the war.

## VI. THE RATE OF MORTALITY.

### (a) *Gross rate of Mortality.*

The following table gives the rate of mortality in the cases of tetanus treated in home hospitals.

Table IV.

*Rate of Mortality in cases of Tetanus occurring in England.*

Number of cases	Recovered	Died	Rate of mortality per cent.
1437	936	501	34·8

Twenty-one cases are deducted from the 1458 as they undoubtedly died from other causes. It is therefore impossible to place them either among the recoveries or the deaths. The rate of mortality among cases of tetanus treated in England is 34·8 per cent. The cases in France have a higher rate since the severe acute cases with a short incubation would naturally be treated there. 715 cases up to the present have been reported from France; 510 of these died; a case mortality of 71·3 per cent. Combining the two sets of figures, there are 2152 cases and 1011 deaths; a rate of mortality of 47·0 per cent. The death rate in the pre-serum days lay about 85 per cent. This lowering of the death rate from 85 per cent. to 47·0 per cent. is doubtless due in great part to the introduction of the prophylactic inoculation of antitetanic serum. It would be much lower than 47·0 per cent. if only cases of wounded men who undoubtedly received a sufficient dose of antitoxin were taken into account. It must be remembered that, so far as prophylactic injections are concerned, we were still practically in the pre-serum days during the first months of the war. Further it is also true that during the whole of the war many cases escaped the preventive dose. Many cases of trench feet, for example, succumbed to tetanus before it was learnt that they must be treated as wounded men.



Doubtless also, owing to the exigencies of war service many wounded men would be marked up as having received a prophylactic injection, who did not in reality receive it.

(b) *The Rate of Mortality at different periods of the War.*

The number of cases of tetanus dealt with at different periods during the war and the rate of mortality are given in Table V.

Table V.

*Rate of Mortality for each year of the War.*

Years	Number of cases	Recovered	Died	Rate of mortality per cent.
1914	182	81	101	55.5
1915	138	60	78	56.5
1916	451	281	170	37.6
1917	376	294	76	20.5
1918	291	204	73	26.3
1919	20	16	3	15.0

From this table it will be seen that the fall in the death-rate has been fairly progressive. This result, to whatever cause it may be due, must be considered as satisfactory.

(c) *The position of the wounds in relation to the incidence and the mortality.*

There were 1458 cases of tetanus; 576 of these had multiple wounds and 882 single wounds.

Of the 576 multiple wounds, 392 recovered and 176 died; a case mortality of 31.0 per cent.

Of the 882 single wounds, 544 recovered, 325 died; a case mortality of 37.4 per cent.

There were 21 cases among the 1458 who died of other diseases. These are not included in the above figures.

The following table gives the position of the wounds in the 882 cases of single wounds.

Table VI.

*Position of wound in cases of single Wounds.*

Position	Number of cases	Incidence per cent.	Recovered	Died	Case mortality
Head, face and neck	29	3.3	24	5	17.2
Trunk ... ..	135	15.2	80	51	37.8
Upper extremities	236	26.7	132	101	43.3
Lower extremities	480	54.4	303	171	36.1

From this table it would appear that wounds of the lower extremity give rise to more cases of tetanus than wounds of other parts of the body, and it might be argued that this was due to the greater risk of earth contamination in these wounds. But to arrive at the truth in this matter it would be necessary

to know the ratio of wounds of the head to those of the other parts. This varies in different wars. What it is in this war has not yet been calculated. In the old wars the average ratio was given as 12, 19, 27 and 42. That is to say there were admitted to hospital 12 cases of head wound, 19 of trunk, 27 of the upper extremities and 42 of the lower extremities for every hundred cases treated.

(d) *The effect of Fractures on the rate of mortality in cases of Tetanus.*

Table VII.

Wound	Number of cases	Recovered	Died	Mortality per cent.
Fracture ...	492	349	143	29·1
No fracture...	945	587	358	37·9

Twenty-one died of other causes, there were among them nine fractures. It has been stated that the presence of a fractured bone in a wound raises the death-rate in cases of tetanus. From the above figures it will be seen that the advantage on the contrary lies with the fractures. It can be readily imagined that the addition of a fracture to a wound might increase the incidence of the disease among wounded men. It is more difficult to understand why the complication of a fracture should heighten the rate of mortality. In regard to the relation of fractures to incidence it is impossible at present to make any statement as the figures are not available.

In the former a fracture should increase the liability of the wounded man to take tetanus, but when once tetanus has supervened it should make little or no difference in the risk whether he has a fracture or not.

(e) *Tetanus occurring after operative interference with the wound.*

Table VIII.

Number of cases	Recovered	Died	Case mortality per cent.
102	67	35	24·5

Among the 1458 cases, 102 are reported to have supervened after a surgical operation. There were 35 deaths, a case mortality of 24·5 per cent.

The Tetanus Committee recommended that when operations are performed, at the site of wounds, even if they are healed, a prophylactic injection of serum should always be given. Among these 102 cases only 7 are stated to have received this prophylactic inoculation before the operation. All seven recovered. There is no record of the effect of prophylactic injections before operation on the incidence of the disease. Only those cases of operation which resulted in tetanus are given. The small number of cases recorded as having received an injection before an operation, would almost go to show that most of those who received the injection did not develop symptoms of tetanus. It has been shown that tetanus bacilli may lie latent for years at the site



of wounds; a prophylactic injection of antitetanic serum before operation would therefore appear to be a reasonable precaution.

Among the cases of tetanus there were some curious examples, which it may be of some interest to record.

				No. of cases	Recovered	Died
Appendicitis	...	...	...	5	2	3
Vaccination	...	...	...	2	1	1
Haemorrhoids	...	...	...	2	1	1
Inguinal hernia	...	...	...	1	—	1
Varicocele	...	...	...	1	—	1
Blister, pricked with dirty needle	...	...	...	1	1	—
No wound discovered	...	...	...	2	2	—
Ulcer of scrotum	...	...	...	1	—	1
A fish-bone stuck in finger while working in garden where fish-manure had been used	...	...	...	1	1	—
Prick of a thistle	...	...	...	1	1	—
Trench feet and frost bite...	...	...	...	38	20	18 (47·3)
Trench feet before 1917	...	...	...	20	5	15 (75)
Trench feet after 1917	...	...	...	18	15	3 (16·6)

At first trench feet were not treated as wounds and no prophylactic serum was given until the resulting death-rate of 75 per cent. Afterwards when serum was given the rate fell to 16·6 per cent.

(f) *On operative interference after tetanus symptoms have appeared.*

Table IX.

Number of cases	Recovered	Died	Mortality per cent.
74	56	18	24·3

The opinion of the Tetanus Committee was that it appeared safer to abstain from surgical interference with a wound after tetanus had appeared. In the 1458 cases, there were 74 which had had such an operation. Of these 56 recovered and 18 died, a case mortality of 24·3 per cent. From this it would appear that operative interference after tetanus symptoms have declared themselves, is not the dangerous procedure it has been supposed to be.

If tetanus symptoms supervene on a wound it would appear to be the best course for the welfare of the patient to thoroughly cleanse the wound and remove any sequestra or scar tissues which may be harbouring the bacillus. But if an operation is decided on it would be well to see that the tissues are well flooded with antitoxin, lest the interference with the wound may let loose on raw surfaces a further quantity of toxin.

(g) *Relation of the Rate of Mortality to the number of days which elapse between the date of wound and the onset of symptoms.*

**Diagram 7** shows that in the 1458 cases dealt with in home military hospitals, if the symptoms of tetanus appeared within ten days of receiving the wound, the rate of mortality was 58·1 per cent., if from the eleventh to

the twenty-second day, 35·3 per cent. The remaining 579 cases with an incubation period of more than twenty-two days, 17·3 per cent. This is the

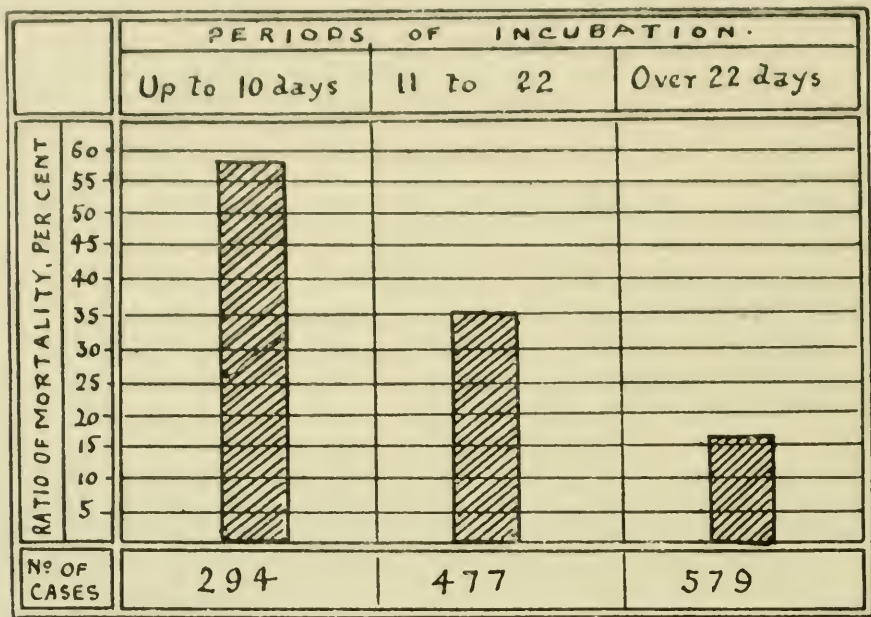


Diagram 7. Death Rate in Cases of Tetanus with short, medium and long Periods of Incubation.

old story; the longer the period of incubation, the lower the rate of mortality. In eighty-seven cases the period of incubation could not be calculated on account of a lack of information in the reports.

VII. PROPHYLACTIC OR PREVENTIVE TREATMENT OF TETANUS.

The anticipation and prevention of tetanus, as in so many other diseases, is by far the most important means of defence. In the first line we may place the proposed league of nations. If there were no wars men would not be called on to stand up to the knees in filthy mud and be torn and lacerated by rough and dirty pieces of shell. It seems a strange and barbarous thing to do, and it is to be hoped that in the course of evolution, mankind will become sufficiently intelligent to find some other way of settling his differences. At the present time, however, it is necessary to find other methods of prevention as man has not yet sufficiently emerged from the savage.

Given then war, and the conditions of warfare which prevailed during the great war, we must expect tetanus to supervene in a certain proportion of the wounded. In the South African war there was little or no tetanus. The wounds were mostly caused by bullets and the veldt was clean. In France the soil was heavily manured and the wounds were largely caused by shell-fire.

The next most important line of defence is the surgical treatment of the wound. This has already been alluded to in the section on the incidence of cases among the wounded. The surgeon's knife, after all is said and done,



is the best means of preventing the occurrence of tetanus after the wound has been inflicted. Dead putrifying tissues is the best culture medium for the anaerobe. At the beginning of the war the treatment of wounds was not thorough enough at the primary operation. It was thought sufficient to wash out the wound and apply an antiseptic. Controversy was acute in regard to the merits of chlorine, common salt, bipp, flavine, etc. It was only when surgical teams were boldly pushed up to the front, and thorough excision of the damaged tissues in the wounds carried out, with primary or primary delayed suture, that any real advance was made in treatment. Anyone who saw these wounds in the base hospitals in France a few days after they had been inflicted, must have been struck by the extraordinary results. One could almost foresee the time when antitetanic serum would no longer be required.

Major Gibson may be quoted in this connexion. "The early incision of wounds began about the middle of 1917, and beyond the very remarkable drop in the incidence of tetanus, due to prophylactic injection, the practice of general excision had a further effect on the incidence of the disease. From 103 per 100,000 in the pre-incision days, the rate of tetanus had fallen to 24 per 100,000 in February 1918. In May there were only 12 cases per 100,000 and 8 per 100,000 in June."

Next in importance as a preventive measure is the prophylactic injection of antitetanic serum. This war has shown that the prophylactic value of antitoxin is beyond all doubt. The first thing in this connexion to be discussed is:—

(a) *The Incidence of Tetanus among protected and unprotected wounded soldiers.*

Some two months after the beginning of the war it was ordered that every wounded man should receive an injection of 500 units of antitetanic serum as soon after he was wounded as possible. That this had a most important influence on the incidence of tetanus among the wounded men is amply proved by Diagram 1. In that diagram it is shown that the ratio of the number of cases of tetanus to the number of wounded was several times as high in September and October as in November and December. In September it was 9 per 1000, in December it fell to 1·4 per 1000. Now this fall was undoubtedly due to the fact that few prophylactic inoculations of antitetanic serum were made until the middle of October. It appears that only a small quantity of the serum was taken out with the Expeditionary Force in August 1914, and this only for purposes of treatment. It was not until the number of cases of tetanus became alarming that steps were taken to secure a large supply and ensure that every wounded man received a prophylactic dose. It was not until about the middle of October that prophylactic inoculation was introduced on anything like an adequate scale, and it was at this time that the remarkable fall in the incidence of the disease took place.

(b) *The Incubation period in non-protected and protected cases.*

The next question is, what effect has the prophylactic inoculation on the length of the incubation period? The following diagram represents the incubation period in the protected and the unprotected. There are 899 protected and 213 unprotected among the 1458. Many cases are not recorded.

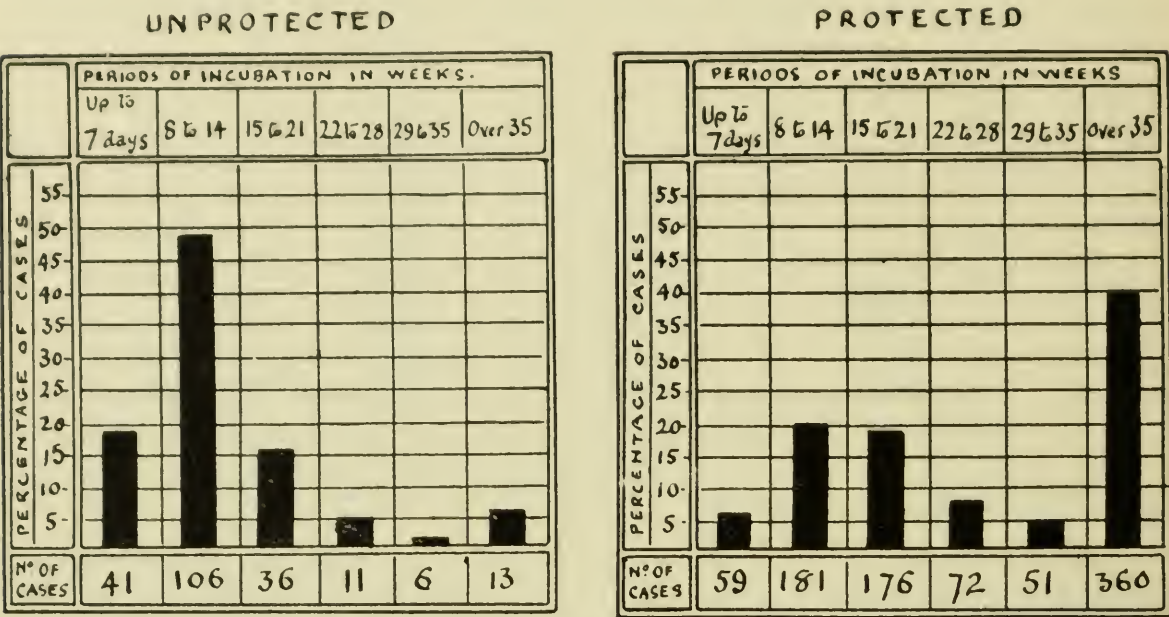


Diagram 8. Comparison of the Period of Incubation in Protected and Unprotected.

Diagram 8 shows that the incubation period is much lengthened in the protected. Only 26.9 per cent. of the protected are attacked by tetanus during the first fortnight, whereas 68.9 of the unprotected are attacked in the same period. Forty per cent. of the protected have an incubation period of more than 35 days, only 6.5 per cent. among the unprotected.

Taking all the cases the average incubation among the protected is 45.5 days, among the unprotected 10.9 days.

It is therefore abundantly manifest that the prophylactic injection of antitoxin lengthens in a marked degree the length of the period of incubation.

(c) *The Rate of Mortality among the protected and the unprotected.*

The following table gives the figures:—

Table X.

*Comparison of the Rate of Mortality in the Protected and Unprotected.*

	Number of cases	Recovered	Died	Rate of mortality per cent.
Protected ... ..	899	676	203	22.5
Unprotected and Unrecorded	559	260	298	53.3

Among the 1458 cases under consideration 899 (61 per cent.) are noted as having received a prophylactic injection of antitetanic serum. Of these



899 protected cases, 676 recovered and 203 died, giving a mortality of 22·5 per cent. In the remaining 559 cases there is no record of prophylactic treatment, although in all probability many of them received it. Of these 260 recovered and 298 died, a mortality of 53·3 per cent.

These figures go to show that the prophylactic inoculation of antitoxin lowers the mortality more than one-half, namely from 53·3 per cent. to 22·5 per cent. Further it must be borne in mind that many wounded men came over from France, especially during the first half of the war, without any records. There was no Field Medical Card at that time. These unrecorded cases have been placed among the unprotected class.

*(d) The effect of multiple prophylactic inoculations on the incidence of tetanus among the wounded.*

At the beginning of the war one prophylactic inoculation was the rule, but in June 1917, it was ordered that four should be given at intervals of seven days, each injection to consist of 500 units. If multiple inoculations are more useful than a single dose, this should show itself in a lowered incidence of tetanus among the wounded. At present it is impossible to make any statement as the data available are insufficient. It is true that the incidence in the latter half of 1917 is lower than in the corresponding part of 1916, as shown in Diagram 1, but whether this is in any way due to the introduction of multiple doses or not it is difficult to say. When all the figures are available it may be possible to see the effect.

Col. Lingard, R.A.M.C., Queen Mary's Military Hospital, Whalley (Lancs.), gives some interesting details bearing on this question. He states that since the order was issued on 17th June 1917, and had time to be introduced and carried into effect, 15,152 surgical patients of the British Expeditionary Force have been admitted to this hospital, without a single case of tetanus developing. He considers this a most encouraging and satisfactory result and that it justifies all the extra expense and labour involved in the multiple inoculations.

At present then the question must be left without a definite answer. One argument in favour of multiple prophylactic inoculations is that if the wounded man has escaped the primary dose owing to the stress of war, this is made good seven days afterwards.

*(e) The effect of one or more than one inoculation on the incubation period.*

**Diagram 9** gives the number of prophylactic inoculations, the number of cases and the average incubation in days. With one inoculation, the average incubation is 38·2 days; with two, 33·6 days; with three, 51 days; and with four or more 83·7 days.

From this it would appear that the incubation period tends to lengthen as the number of inoculations increases; but whether this is due to the multiple injections or not, it is difficult to say.

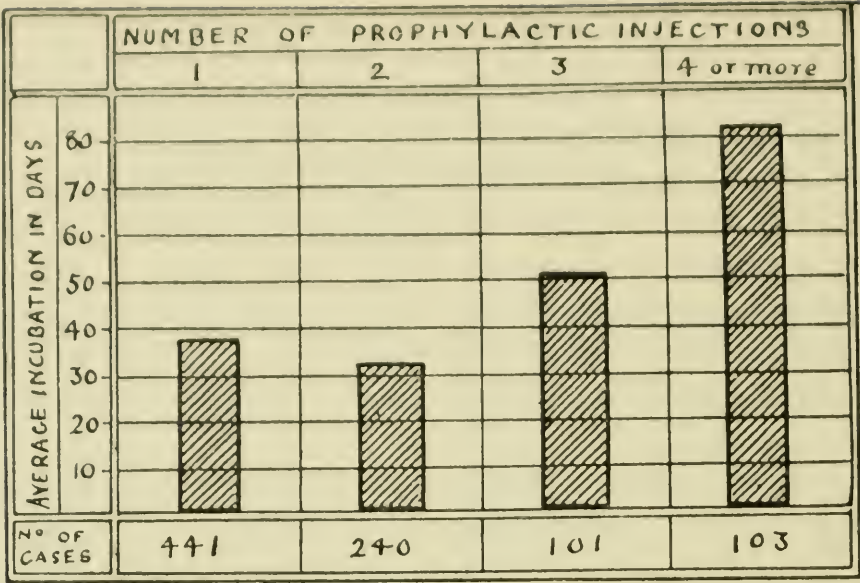


Diagram 9.

(f) *On the effect of one, or more than one, prophylactic inoculation on the rate of Mortality.*

**Diagram 10** represents the effect of one, or more than one, prophylactic inoculation on the death-rate. From it will be seen that wounded men who received one inoculation had a case mortality of 25.1 per cent.; those who received two, 21.3 per cent.; three, 16.5 per cent.; and four or more 7.1 per cent.

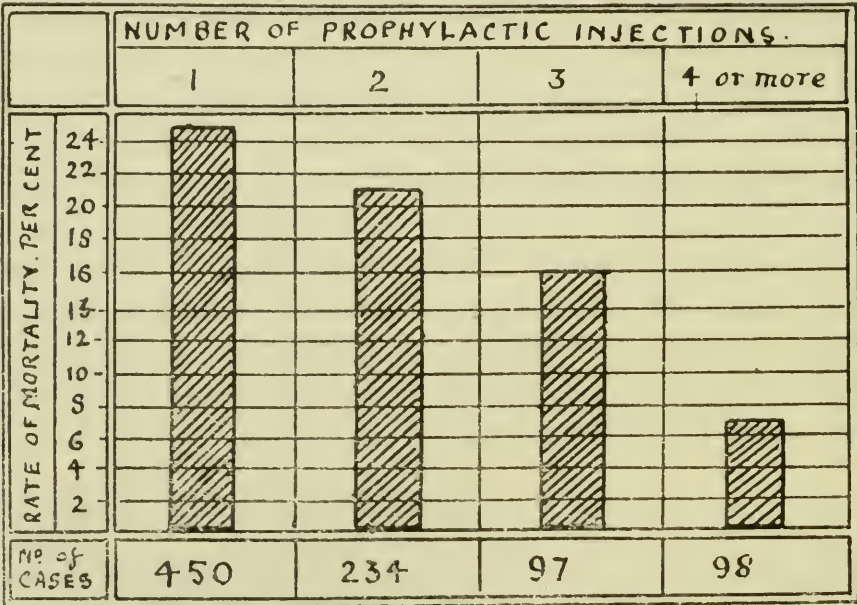


Diagram 10. The effect of one or more prophylactic inoculations on the death-rate.

From these diagrams it may be said that the Tetanus Committee were justified in recommending multiple prophylactic injections. The incubation period appears to be lengthened and the death-rate diminished. Whether this is really due to the increase in the number of inoculations, or merely to



the fact that the cases which receive three or four injections are naturally the milder, more chronic, slower cases of the disease, with a lower rate of mortality, it is impossible to say. But although it must be confessed that the value of this procedure is by no means proved by these figures, it will probably be safe to recommend that multiple inoculations should be continued until more evidence is accumulated.

(g) *What effect, if any, has the time of inoculation on the rate of mortality?*

In regard to what influence promptitude in giving the prophylactic inoculations has on the rate of mortality, the following table gives all the information available from the 1458 cases.

Table XI.

*The effect of the time of inoculation on the rate of Mortality.*

Number of cases	Prophylactic inoculation	Recovered	Died	Mortality per cent.
611	On day of wound	467	144	23·5
140	One day after wound	111	29	20·7
106	Two days after wound	79	27	25·4

From this table it would appear that it is better to be inoculated the day after receiving the wound than on the day of the wound. But after all there is not much difference in the three rates and it probably does not make much difference in the death-rate whether the first prophylactic inoculation is given on the day of wound or a day or two after. At least this is what the figures would seem to show. There probably is some effect on the incidence of the disease among the wounded, and it would appear to be self-evident that the sooner the prophylactic is given after the wound is received, the better.

(h) *On the results obtained from the Increase of the Primary Prophylactic dose from 500 to 1500 units.*

On the recommendation of the Adviser in Pathology to the British Expeditionary Force in France, the primary prophylactic dose was changed from 500 to 1500 units. This change took place about the beginning of August 1918. The following table shows the amount of the primary prophylactic dose, the number of cases, the average period of incubation, and the rate of mortality.

Table XII.

*The effect on the rate of Mortality of the amount of the primary dose of serum.*

Primary prophylactic dose	Number of cases	Average length of incubation in days	Rate of mortality per cent.
500 units	238	38·6	22·4
750 „	140	42·1	16·4
1000 „	40	59·0	10·5
1500 „	106	25·0	27·4

This table is given for what it is worth. From it will be seen that 238 cases of tetanus which received a primary prophylactic dose of 500 units had an incubation period of 38·6 days and a death-rate of 22·4 per cent.; whereas 106 men who are stated to have received a primary dose of 1500 units had an incubation period of only 25 days and a death-rate of 27·4. The deduction is that the primary injection of 500 units as recommended by the Tetanus Committee, should be continued, as it seemed to be quite as efficacious as the 1500 units. This will mean economy, in money, space and transport. Further it is probable that 3 c.c. of horse serum are better borne and give rise to less irritation than 9 c.c.

It must also be remembered that four prophylactic doses are given at intervals of seven days. If only one dose is given it is possible that a larger primary dose than 500 units might be given with advantage, but there is no proof of this.

(i) *The addition to the antitetanic serum of the antitoxins of other anaerobes found in wounds.*

It may be useful to record here that attempts were made towards the end of the war, to add to the antitetanic serum the antitoxins of *B. welchii*, *Vibrio septique* and *B. oedematiens*. An account of this is given in the fourth edition of the Memorandum on Tetanus and also in *War Medicine*, December 1918.

It will be sufficient to say here that no satisfactory or practical results came of these attempts, as the war ended before the methods were at all perfected. If this attempt is made at any future time it would be advisable to make the experiment independently of the antitetanic serum lest the latter be brought into disrepute and we lose the substance in snatching at the shadow.

#### VIII. THERAPEUTIC OR CURATIVE TREATMENT OF TETANUS.

This is undoubtedly a difficult subject to be dogmatic upon. The curative treatment of tetanus by carbolic acid, magnesium sulphate and other salts may be dismissed with the remark that in home hospitals they proved a failure and soon dropped out of the running. The only specific therapeutic treatment is by the injection of tetanus antitoxin. It has been proved up to the hilt that the prophylactic inoculation of antitoxin is of very great value; lowering the incidence, lengthening the period of incubation and lessening the death-rate. But when an attempt is made to appraise the value of antitoxin, given after the symptoms of tetanus have declared themselves, great difficulty is met with. Wide differences of opinion are held, many holding that if given early and in sufficient quantity it acts powerfully for good; others doubting its usefulness but hesitating to discard it altogether. The latter argue that as it is the only rational specific drug against tetanus in



our possession it would be wrong to withhold it in such a fatal disease as tetanus. They think there is an off chance that it may turn the scale in favour of the wounded man.

The experimental evidence is in favour of the antitoxin as a curative agent. Prof. Sherrington has shown that serum treatment of experimental tetanus in monkeys shows a recovery rate in a series of 25 animals of 44 per cent. The monkeys were inoculated with a dose of tetanus toxin more than eight times the minimum lethal dose. The antitetanic serum was not brought into play until twelve to sixty hours after the symptoms of tetanus had declared themselves. It is true that there is a great difference in tetanus in man and experimental tetanus in the monkey. In the one case there is present the living tetanus bacillus, in the other a weighed quantity of the dead poison. But the fact still remains that here we have animals, suffering from definite symptoms of tetanus, who would undoubtedly all die in a very short time if untreated, receiving a single dose of antitoxin with the result that half of them recover.

In regard to the statistical proof of the value of antitoxin as a curative agent, it is doubtful if any truth can be arrived at by the study of the figures at our disposal. There is no uniformity in the treatment of tetanus in man. The men who suffer are also, as a rule, suffering from other grievous maladies—wounds, fractures, septicaemias, pneumonias, haemorrhages, heart failures, etc., so that if the man dies it is impossible in many cases properly to fix the blame.

Capt. Golla, a member of the Tetanus Committee, has compared the results obtained from the use of therapeutic serum in this war with those of pre-serum days. He is of opinion that the rate of mortality in cases of tetanus in this war which did not receive a prophylactic injection of antitoxin but did receive therapeutic treatment, approaches very closely to the rate of mortality in pre-serum days. In other words, it would appear from his figures that the therapeutic use of serum is of little or no practical value in treatment. It is taught at the present time that tetanus toxin which has been taken up and fixed by the nerves or nerve cells is inaccessible to antitoxin. If a lethal dose has been taken up by the nerves and is travelling towards the nervous centres, before the serum treatment is begun, then no amount of antitoxin will save the patient.

But in spite of these statistical considerations, and in view of the experimental results, it is clear that medical officers will continue for the present to give a case of tetanus the benefit of the doubt and use antitetanic serum therapeutically. As Ransom states, it may neutralise some of the free toxin in the blood and lymph, and prevent it ultimately entering the nervous system and causing death, when the toxin already admitted through the motor nerves is not sufficient to do so.

It remains then to decide as to the best route and the best dosage.

The total quantity of antitetanic serum issued to the army during the

war was 11,258,125 doses of 500 units of low potency serum and 27,549 doses of 8000 units of high potency serum.

(a) *What is the best route for the administration of antitetanic Serum?*

No answer to this question can be given from a study of the figures available for this report. Experience during the war does not seem to have led to any uniformity of practice. In one school a large single dose of some 30,000 units of antitoxin given intravenously is the method used. In another the subcutaneous route is chosen, and as much as 100,000 units given during the first 24 hours. In yet a third the intramuscular is considered the best. The Tetanus Committee on the other hand are of opinion that in acute general tetanus the best method of treatment lies in the earliest possible administration of large doses of antitetanic serum by the intrathecal route. The Committee agrees that the intravenous route is an excellent and rapid method of giving serum, but thinks that the danger from anaphylactic shock renders it less useful.

As there did not seem to be any hope of proving which is the best route from the figures at their disposal the Committee instituted a series of animal experiments. Prof. Sherrington carried these out at Oxford, and the result is given in the following table.

Table XIII.

*On the effect of route on the administration of antitetanic serum.*

Route	Number of animals used	Number of deaths	Number of recoveries
Subcutaneous ...	25	23	2
Intramuscular ...	25	22	3
Intravenous ...	25	18	7
Lumbar subdural	25	11	14
Bulbar subdural	20	7	13
Cerebral subdural	10	10	—

It will be seen from the above table that the results are indubitably in favour of the intrathecal route.

Out of the total of 1458 cases of tetanus treated in home hospitals, 1389 had injections of antitetanic serum after the onset of the symptoms. Of these 923 recovered and 466 died, a rate of mortality of 33·5 per cent.

The following table gives the various routes employed in the administration of antitetanic serum in the 1389 cases. In the remaining 69, 43 had no therapeutic injection of any kind, 21 died of other diseases and in 5 the route is not specified. This tabular statement is given in order to place on record the methods used during the war. It is useless for the purpose of obtaining evidence as to the best route.



Table XIV.

Cases were treated by injections of serum	Cases	Recovered	Died	Mortality per cent.
Subcutaneous alone ... ..	248	129	119	47·9
Intramuscular alone ... ..	133	119	14	10·0
Intravenous alone ... ..	33	16	17	51·5
Intrathecal alone ... ..	43	19	24	56·5
Subcutaneous and intramuscular	85	76	9	10·6
Subcutaneous and intravenous	49	23	26	53·0
Subcutaneous and intrathecal ...	163	88	75	46·0
Intramuscular and intravenous	28	18	10	35·7
Intramuscular and intrathecal	250	177	73	29·2
Intravenous and intrathecal ...	24	11	13	54·1
Subcutaneous, intramuscular and intravenous	9	3	6	66·6
Subcutaneous, intramuscular and intrathecal	205	161	44	21·4
Subcutaneous, intravenous and intrathecal	45	31	14	31·1
Intramuscular, intravenous and intrathecal	47	30	17	36·1
Subcutaneous, intramuscular, In- travenous and intrathecal	27	22	5	18·5

*(b) What is the best dosage.*

The Tetanus Committee recommended in their Memorandum that in the treatment of acute general tetanus large doses should be given. The term "large dose" is not very definite. By it the Committee meant about 24,000 units in 24 hours. Towards the end of the war a circular was sent round by the British Medical Authorities in France recommending much larger doses. The circular states that the treatment of generalised tetanus by means of antitoxin had not so far given encouraging results, and that in a recent series of 60 cases the case mortality was 71·2 per cent. The dosage of 24,000 units on the first days, as recommended by the Tetanus Committee, was looked upon as too small, and a dosage three times as large was advocated. Three cases are reported in which recovery took place after very large doses. The first received 64,000 units during the first 24 hours, given in three intrathecal, one intravenous and four subcutaneous injections. Eight different injections in the first 24 hours and all of 8,000 units. This may be necessary, but it is difficult to picture such a quantity of toxin circulating in the blood and lymph as to require such a huge quantity of antitoxin for its neutralisation. The first two cases are reported to have had severe serum rashes, and one is reported to have had a moderate anaphylactic shock. It surely cannot be necessary to give such large and numerous doses. If symptoms of tetanus appear it seems necessary with our present knowledge to inject antitoxin. Animal experiments show that the intrathecal route is the most efficacious. One would therefore conclude that a dose of 20 c.c. of high potency serum, containing 16,000 units, given intrathecally on the first and second days, supplemented and continued by intramuscular and subcutaneous injections

would be sufficient to keep the fluids of the body amply supplied with antitoxin.

In regard to the quantities of antitoxin used therapeutically in home hospitals during the war the following two tables give approximately the total amount of serum given, and the average daily amount.

Table XV.

*The Total quantity of antitoxin given in Home Military Hospitals.*

Out of the 1381 cases		Recovered	Died	Mortality per cent.
15 received	1,000 units or under	7	8	53.3
156	1,001 ,, 5,000 ,,	79	77	49.3
144	5,001 ,, 10,000 ,,	64	80	55.5
106	10,001 ,, 15,000 ,,	65	41	39.6
84	15,001 ,, 20,000 ,,	49	35	41.6
166	20,001 ,, 30,000 ,,	104	62	37.3
114	30,001 ,, 40,000 ,,	84	30	28.0
180	40,001 ,, 60,000 ,,	123	57	31.6
202	60,001 ,, 100,000 ,,	155	47	23.1
158	100,001 ,, 200,000 ,,	141	17	10.7
32	200,001 ,, 300,000 ,,	26	6	18.7
9	300,001 ,, 400,000 ,,	8	1	11.1
9	400,001 ,, 500,000 ,,	9	—	—
4	500,001 ,, 600,000 ,,	4	—	—
2	600,001 ,, 900,000 ,,	2	—	—

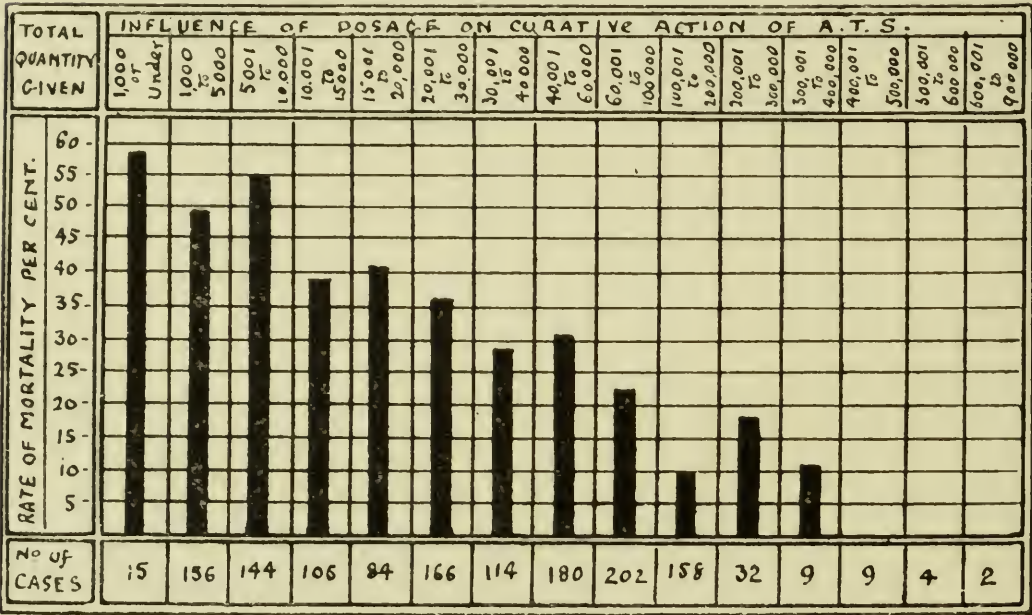


Diagram 11. Influence of dosage on curative action of antitoxin.

It appears from **Diagram 11** that the greater the amount of serum given the lower the mortality. This probably merely means that the longer the man lived the more serum he received. The short acute cases died before they had time to receive a large quantity. Some of the men received very large quantities indeed. One man for example received 150,000 units daily for



3 days; an average of 45,000 for 20 days; the grand total being 900,000 units. If ordinary serum was used at 1500 units to 10 c.c., then the man received 6 litres of horse serum in 20 days. As each dose of 500 units costs one shilling then this man's treatment cost £90 in serum alone. This huge quantity was all given intramuscularly; the man is reported to have had a slight serum rash, and that he recovered.

Table XVI.

*The average daily quantity of antitoxin given during the attack.*

Out of the 1381 cases		Recovered	Died	Mortality per cent.
36 received	1,000 units or under	24	12	33.3
487	1,001 „ 5,000 „	308	179	36.1
363	5,001 „ 10,000 „	280	83	22.8
194	10,001 „ 15,000 „	145	49	25.2
114	15,001 „ 20,000 „	78	36	31.5
109	20,001 „ 30,000 „	48	61	55.9
47	30,001 „ 40,000 „	23	24	57.0
25	40,001 „ 60,000 „	11	14	56.0
6	60,001 „ 100,000 „	3	3	50.0

**Diagram 12** gives the average daily dose. Here an opposite condition prevails. The larger the dose the higher the mortality. This probably merely means that the more severe cases received larger daily doses than the less severe cases.

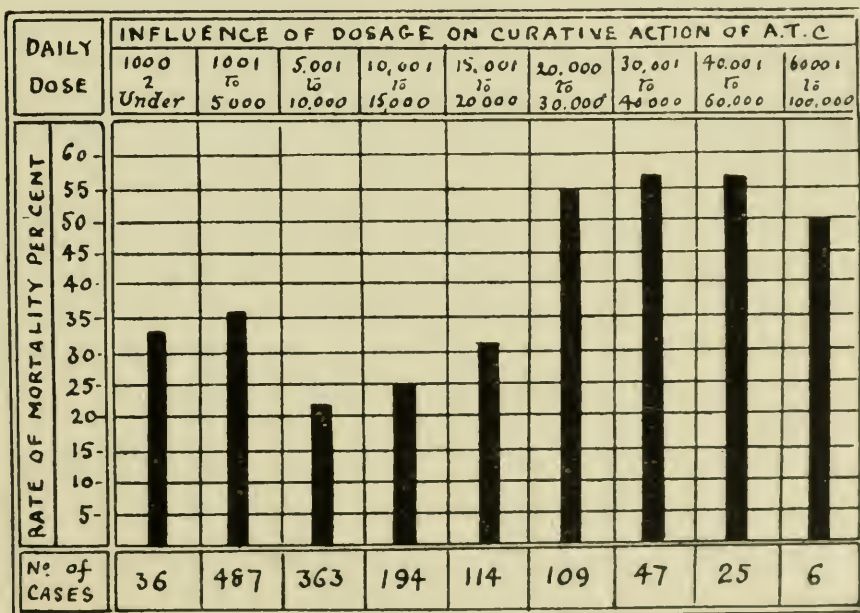


Diagram 12. The average daily dose of antitoxin in home military hospitals.

It is quite evident that no deduction as to the value of antitetanic serum as a therapeutic agent can be made from these premises. At present then the position seems to be this: antitoxin has no power of neutralising toxin in the nervous system. If a fatal amount has been absorbed then no amount of antitoxin will save the man's life. If there is any free toxin circulating in

the blood or lymph the antitoxin may neutralise it and so possibly prevent further mischief. If then a fatal amount has not been absorbed by the nerve cells, the injection of antitoxin may be of use. By animal experiment it appears to be proved that the intrathecal route is the best. Therefore the Tetanus Committee in the fourth edition of the Memorandum on Tetanus recommended antitoxin intrathecally at the first sign of tetanus.

#### IX. TYPES OF TETANUS BACILLI.

Major Tulloch, a member of the Tetanus Committee, worked with the greatest industry in order to obtain pure cultivations of the tetanus bacilli with a view to separating and studying the different strains. By means of agglutination tests he has separated them into four types. It was expected that each type would be specific to its own serum, but this was found not to be the case. Antitoxin prepared from any one of the types is stated to neutralise the toxins of all the types. It appears that in the past, tetanus antitoxin has been as a rule prepared from Tulloch's type 1 bacillus, but now as the result of his work it is usual in England to inject the horses with toxins from all four types. This subject is admittedly in its infancy and Tulloch's papers should be consulted for fuller information.

#### X. ANAPHYLAXIS.

##### *Anaphylactic shock after prophylactic injections.*

These injections consist of 3 c.c. of horse serum if 500 units are given; of 9 or 10 c.c. if 1500 are given:—serious symptoms resulting from these small subcutaneous injections are exceedingly rare, but occasionally severe shock may follow the injection of even the smaller of these doses.

In England alone, during the war, it is probable that some two millions of prophylactic doses of antitetanic serum have been given. Out of this huge number only eleven cases of shock have been reported. All eleven cases recovered. No doubt these cases appear very alarming when they occur, but they are so rare that they may be looked upon as negligible.

##### *Anaphylactic shock following the therapeutic use of serum.*

Since the beginning of the war, 49 cases of anaphylactic shock have been notified (3·5 per cent.). There were 12 deaths (0·8 per cent.). Fifteen cases were reported as severe, 6 as moderate, and 16 as mild.

Of the 49 cases of shock, 17 were caused by intrathecal injections, 14 by intravenous, 9 by intramuscular and 2 by subcutaneous. In 7 it is doubtful which injection caused the shock. Of the whole number of cases of tetanus, 757 received intrathecal, 232 intravenous, 736 intramuscular and 819 subcutaneous injections.

It follows then that 2 per cent. of the cases of shock followed the intrathecal injections, 6 per cent. the intravenous, 1·2 per cent. the intramuscular and



0·2 per cent. the subcutaneous route of injection. It is evident from these figures that the most dangerous route for the therapeutic injection of anti-tetanic serum is the intravenous, and that the Tetanus Committee were justified in not recommending it.

From this it will be seen that anaphylactic shock after therapeutic injections of antitetanic serum is by no means a rare phenomenon, and markedly reduces the questionable usefulness of therapeutic serum.

## REFERENCES.

*Being a list of Papers written by members of the Tetanus Committee and others, which may be consulted.*

- A.M.D. 2, War Office (Issued by) (1917). *Memorandum on the use of curative sera.*  
 A.M.D. 2, War Office (Issued by) (1917). *Notes on the nursing of tetanus.*  
 A.M.D. 2, War Office (Issued by) (1918). *Memorandum on Tetanus* (4th Edition).  
 ANDREWES, F.W. (5. v. 1917). On the Intrathecal route for the administration of tetanus antitoxin. *Lancet.*  
 BARNES, STANLEY (17. III. 1917). Tetanus relapse after a trivial operation. *Brit. Med. Journ.*  
 BOWLBY, SIR ANTHONY (2. VIII. 1919). A sketch of the growth of the Surgery of the front in France. *Brit. Med. Journ.*  
 BRUCE, SIR DAVID (23. x. 1915). An analysis of cases of tetanus treated in Home Military Hospitals from August 1914 to August 1915. *Lancet.*  
 — (2. XII. 1916). 2nd analysis of cases of tetanus treated in Home Military Hospitals from August 1, 1915 to July 31, 1916. *Lancet.*  
 — (30. VI. 1917). 3rd analysis of cases of tetanus treated in Home Military Hospitals from August to October 1916. *Lancet.*  
 — (15. XII. 1917). 4th analysis of cases of tetanus treated in Home Military Hospitals from October to December 1916. *Lancet.*  
 — (22. XII. 1917). 5th analysis of cases of tetanus treated in Home Military Hospitals from December 1916 to March 1917. *Lancet.*  
 — (16. III. 1918). 6th analysis of cases of tetanus treated in Home Military Hospitals from March to June 1917. *Brit. Med. Journ.* (Summary only).  
 — (12. x. 1918). 7th, 8th and 9th analyses of cases of tetanus treated in Home Military Hospitals from June 1917 to April 1918. *Brit. Med. Journ.* (Summary only).  
 — (XI. 1917). Tetanus in Home Military Hospitals. Analysis of 1000 cases. *Trans. Soc. Trop. Med. and Hyg.*  
 — (27. I. 1917). Note on the incidence of tetanus among wounded soldiers. *Brit. Med. Journ.*  
 — (13. I. 1917). Importance of early prophylactic injection in trench foot. *Brit. Med. Journ.*  
 — (5. v. 1917). Intramuscular versus the Intrathecal route in the treatment of tetanus by the injection of antitoxin. *Lancet.*  
 — (XII. 1918). Tetanus. *War Medicine.*  
 BULLOCK, W. E. AND CRAMER, W. (15. v. 1919). On a new factor in the mechanism of bacterial infection. *Proc. Roy. Soc. Ser. B.* xc. 513.  
 CUMMINS, S. L. AND GIBSON, H. GRAEME (1. III. 1919). An analysis of cases of tetanus occurring in the British Armies in France between November 1, 1916 and December 31, 1917. *Lancet.*

- DEAN, H. R. (5. v. 1917). Report on twenty-five cases of tetanus. *Lancet*.
- FOSTER, M. (2. II. 1917). Delayed tetanus in connection with fractures. *Brit. Med. Journ.*
- GIBSON, H. G. (15. IX. 1917). A note on the reflexes in tetanus. *Lancet*.
- GIBSON, H. GRAEME (XII. 1918). Discussion on tetanus. *War Medicine*, p. 745.
- GOADBY, SIR K. (X. 1915). A report on the injection of antitetanus serum in tetanus cases. *Journ. Roy. Army Corps*.
- (25. v. 1918). The bacterial flora of war wounds. *Brit. Med. Journ.*
- GOLLA, F. (5. v. 1917). A comparison of subcutaneous with intravenous and intrathecal administration of tetanus antitoxin in experimental tetanus. *Lancet*.
- (29. II. 1918). Analysis of recent tetanus statistics. *Lancet*.
- LEISHMAN, SIR W. B. AND SMALLMAN, A. B. (27. I. 1917). An analysis of recent cases of tetanus in the B.E.F. *Lancet*.
- MACCONKEY, A. T. (10. x. 1914). Tetanus: its prevention and treatment by means of antitetanic serum. *Brit. Med. Journ.*
- (11. XII. 1915). The prophylaxis of tetanus: a Summary. *Brit. Med. Journ.*
- (6. I. 1917). Keeping qualities of therapeutic serum. *Brit. Med. Journ.*
- MACCONKEY, A. T. AND ZILVA, S. S. (18. III. 1916). Iodine in tetanus. *Brit. Med. Journ.*
- MACCONKEY, A. T. AND HOMER, A. (17. II. 1917). On the passive immunity conferred by a prophylactic dose of antitetanic serum. *Lancet*.
- (III. 1917). A plea for a collective study of tetanus. *Journ. Roy. Army Corps*.
- RANSOM, F. (22. XII. 1917). A modern view of tetanus. *Lancet*.
- SHERRINGTON (29. XII. 1917). Observations with antitetanus serum in the monkey. *Lancet*.
- TULLOCH, W. J. (2. IV. 1918). The isolation and serological differentiation of *B. tetani*. *Proc. Roy. Soc. Ser. B*, xc. 145.
- (1. VI. 1918). Concomitant anaerobic injections in tetanus. *Brit. Med. Journ.*
- (1. VII. 1919). The distribution of the serological types of *B. tetani* in wounds of men who received prophylactic inoculation and a study of the mechanism of infection in and immunity from tetanus. *Proc. Roy. Soc. Ser. B*, xc. 529.
- (29. VIII. 1919). Report on bacteriological investigations undertaken on behalf of the War Office Committee for the study of Tetanus. *Journ. Hyg.* xviii. 103–202.
- WORSTER-DROUGHT, C. C. (I. 1918). Observations on a severe case of tetanus treated with repeated intrathecal injections of antitoxin. *Journ. Roy. Army Corps*.
- (1. VI. 1918). A case illustrating an extreme modification of local tetanus. *Lancet*.



A STUDY OF THE SEROLOGICAL REACTIONS OF  
MENINGOCOCCI AND AN ACCOUNT OF THE METHOD  
OF PREPARATION OF ANTI-MENINGOCOCCUS SERUM.

By A. STANLEY GRIFFITH, M.D.<sup>1</sup>

(*From the Field Laboratories, University of Cambridge.*)

(With 2 Charts.)

CONTENTS.

	PAGE
Introduction . . . . .	33
<b>Part I. Serological reactions of meningococci . . . . .</b>	<b>34</b>
Section 1. Colonel Gordon's strains . . . . .	35
Summary of results with the first series of meningococci . . . . .	42
Section 2. Local Government Board strains . . . . .	43
Summary of results with the second series of meningococci . . . . .	48
Section 3. Unselected strains . . . . .	49
Summary of results with the third series of meningococci . . . . .	50
Tests with agglutinating sera from horses . . . . .	50
Tests with agglutinating sera from goats . . . . .	53
Variability of serological results . . . . .	54
Varieties of meningococcus antigen . . . . .	55
Summary and conclusions . . . . .	56
<b>Part II. The preparation of anti-meningococcus serum . . . . .</b>	<b>57</b>
Preliminary experiments . . . . .	58
Method of Amoss and Wollstein . . . . .	59
Modification of above method . . . . .	62
Immunisation of horses with dried cocci . . . . .	65
Tests of the potency of anti-meningococcal serum . . . . .	65
References . . . . .	67

INTRODUCTION.

THE work on the meningococcus described in this report was begun in 1916 in connection with the measures instituted by Lieut.-Colonel M. H. Gordon, C.M.G., for the preventive control of cerebro-spinal fever in the Army.

These measures required large quantities of monovalent agglutinating sera for the identification of the four types of meningococci defined by Colonel Gordon. The preparation of the sera from rabbits was carried out at the Central C.-S. F. Laboratory and on account of the great demand entailed much labour. With a view to helping to meet this demand it was arranged by the Medical Research Committee, at Colonel Gordon's request, that I should prepare agglutinating sera from horses against the four types of meningococci. Unfortunately the horse sera did not prove sufficiently specific for differential purposes and the work was discontinued. Later on, the immunisation of horses was resumed with the object of testing different methods of preparing therapeutic sera.

While the above work on horses was in progress I took the opportunity to study the serological reactions of the various strains of meningococci

<sup>1</sup> Report to the Medical Research Committee.

which had come into my possession, and to compare the relative values of agglutinating sera from different species of animals (horse, goat and rabbit) for the differentiation of meningococci. The results of these serological tests are detailed in Part I of this report.

Part II of my report deals with the methods of preparation of anti-meningococcus serum for therapeutic purposes.

The work was done at the Field Laboratories, University of Cambridge.

## PART I.

### SEROLOGICAL REACTIONS OF MENINGOCOCCI.

*Source of strains.* The strains of meningococci used in this investigation were obtained from three different sources and each group of strains is considered in a separate section.

Colonel Gordon supplied me with 44 strains of meningococci, derived from military cases of cerebro-spinal fever, which had previously been identified either by the agglutination test alone, or by the agglutination and absorption tests with one or other of his four types.

The second series was sent to me by Dr F. Griffith from the Local Government Board's laboratory and comprised 31 strains. These were to some extent selected, many being strains which had been found to have a limited capacity for absorbing agglutinins. All were derived from the cerebro-spinal fluid of cases of meningitis which had occurred during the epidemic prevalence of cerebro-spinal fever.

The third series of 18 unselected strains from adult cases of cerebro-spinal fever were obtained from Mr H. W. C. Vines and Dr W. M. Scott.

*Preparation of Agglutinating Sera.* All the sera used in the investigation of the serological characters of the different groups of strains were prepared (with the exception of one from a goat) from rabbits with representative strains from each of Gordon's four types. The rabbits were immunised by the intravenous injection of cultures grown on glucose agar. The inoculations were made with stock suspensions and only occasionally were living cultures used. The initial dose was 5 or 10 mgrm., and this was gradually increased, at five to ten days' intervals, to a maximum of 80 mgrm. The majority of the rabbits were found to give a good agglutinating serum after from three to five weeks, when the dose had reached 40 or 50 mgrm. When the titre of a serum was satisfactory the rabbit was bled and the serum preserved with 0.5 per cent. carbolic acid.

The methods of immunising horses and goats are described in the sections dealing with the results of serological tests with the sera of these animals.

*Technique of agglutination and absorption tests.* Cultures for these tests were grown on glucose agar at 37° C. for 24 hours. The growth was scraped off, put into a previously tared sterile test tube and weighed. Sufficient 0.5 per cent. carbolic acid in 0.85 per cent. salt solution was added to form a suspension, 1 c.c. of which contained 20 mgrm. of culture. The tubes were then



heated in a water bath at 65° C. for half-an-hour. This constituted the stock suspension and was used in that strength for absorption tests. For agglutination tests the stock suspension was diluted so that 1 c.c. contained 2 mgrm. of culture. The agglutination tests were made in small tubes measuring 3" ×  $\frac{1}{2}$ ". Into each of these 0.5 c.c. of the serum dilution and 0.5 c.c. of the suspension of cocci were pipetted and the tubes, after being corked, were put into a 55° C. incubator for 24 hours; at the end of 24 hours readings were taken.

The greatest concentration of serum in a mixture was 1 in 100. The end point chosen was the highest dilution at which the cocci were completely clumped and sedimented, the supernatant fluid being quite clear or showing only the faintest trace of cloudiness. Each figure in the tables represents this end point. When there was well-marked clumping at 1 in 100, but the fluid remained cloudy, this result is represented by the + sign. The symbol (+) represents a slighter degree of agglutination. When there was no clumping, or only a trace at 1 in 100, the — sign is used.

For absorption tests equal quantities of the stock suspension of the coccus (20 mgrm. per c.c.) and the diluted serum were incubated at 55° C. over-night in centrifuge tubes. Usually the serum was diluted 1 in 25, but when the titre of the serum was very high a 1 in 50 dilution of the serum was employed. Thus, each cubic centimetre of a mixture contained 10 mgrm. of cocci in a 1 in 50, or a 1 in 100 dilution of the serum. After centrifuging, the supernatant fluid was used for agglutination tests on the homologous coccus, and each absorbed serum was also tested on the absorbing coccus.

#### SECTION 1.

##### Colonel Gordon's strains.

This series comprises 44 strains which had been classified as follows: 18 Type I, 17 Type II, 4 Type III and 5 Type IV. One of the strains in the Type I sub-group was found by me on investigation by absorption tests to be not a Type I but a Type III coccus. Gordon (1917) has stated that some of his early strains provisionally classified as Type I would probably be found on application of the absorption test to be Type III strains, and this statement has been confirmed by Tulloch (1917). As this was no doubt one of those strains it has been transferred to the Type III sub-group. The amended classification of the strains is therefore as follows: 17 Type I, 17 Type II, 5 Type III and 5 Type IV.

The object of the investigation was to ascertain whether Gordon's classification of the strains would hold good with sera prepared from some of them in a different laboratory.

Monovalent agglutinating sera were prepared by me from rabbits with strains from each of the four types of cocci, and altogether 15 of the 44 strains were used for this purpose.

The results of agglutination and absorption tests with the sera from each type of coccus will now be considered in detail. The agglutination results are set out in Tables I (a) and I (b).

Table I (a). *Simple agglutination tests upon Gordon's strains of meningococci with sera prepared from members of each of the four types.*

Designation of strains	Type I sera			Type III sera				Type II sera			Type IV	
	"Cou" serum titre 1-800	"All" serum titre 1-1600	"Litt" serum titre 1-800	"Bunt" serum titre 1-3200	"Bunt" serum titre 1-800	"Mac" serum titre 1-1600	"Mer" serum titre 1-1600	"D" serum titre 1-1600	"Off" serum titre 1-3200	"Fos" serum titre 1-3200	"Harr" serum titre 1-100	"Hic" serum titre 1-400
Type I—												
Cou	800	3200	800	200	400	100	200	200	800	800	400	400
All	200	1600	800	...	800	200	100	200	400	400	400	100
Litt	200	800	800	400	1600	800	800	200	1600	400	400	800
Win	800	1600	800	200	200	100	200	200	400	100	100	100
Sher	800	3200	800	200	400	200	200	200	800	400	200	400
Kn	800	3200	800	200	200	400	200	200	400	200	200	100
Lis	800	1600	800	400	...	200	...	...	...	...	...	...
Mit	800	1600	800	200	+	200	100	100	200	400	100	200
How	200	1600	800	+	+	200	(+)	100	200	100	(+)	(+)
Bal	400	1600	1600	...	800	200	200	200	800	200	100	200
Fox	400	1600	800	200	800	200	200	200	400	200	200	200
Sco	...	1600	400	100	400	200	100	100	200	200	100	100
Char	400	3200	800	200	800	400	200	400	400	400	200	200
Far	200	1600	800	800	400	800	800	400	800	400	400	200
Mif	400	1600	800	—	200	100	200	100	(+)	100	100	(+)
Pay	...	3200	800	...	800	200	400	100	...	400	...	...
Jor	1600	3200	1600	400	800	200	100	...	...	...	...	...
Type III—												
Mac	200*	200	200	1600	1600	1600	1600	100	1600	200	400	100
Bunt	+	200	400	3200	800	1600	...	100	800	200	400	200
Cha	...	100	100	...	1600	800	800	200	800	400	400	400
Mer	...	200	100	1600	1600	1600	1600	200	800	200	800	200
Jac	—	(+)	100	...	800	800	1600	100	...	100	...	...
Type II—												
McP	—	100	200	100	400	400	200	200	800	800	400	400
Wat	—	(+)	100	(+)	200	—	—	1600	800	3200	800	400
Mi	(+)	200	200	400	1600	400	800	1600	3200	3200	800	200
Wy	—	100	200	400	800	400	400	800	1600	3200	1600	200
Glid	—	(+)	400*	(+)	400	(+)	100	1600	3200	3200	800	400
Mat	...	(+)	+	—	(+)	—	(+)	800	800	800	200	(+)
Off	—	200	(+)	...	800	...	100	800	3200	6400	800	400
Cu	—	+	100	100	800	...	400	1600	1600	3200	800	400
New	—	200	200	...	800	...	100	800	3200	3200	1600	200
Mor	...	(+)	100	(+)	+	...	(+)	1600	800	1600	400	400
Harr	—	(+)	(+)	...	+	...	+	400	800	800	100	400
Fos	—	200	—	...	400	...	+	800	800	3200	800	400
Wal	...	+	(+)	...	400	800	200	1600	1600	6400	800	100
Wet	...	+	(+)	...	400	...	100	1600	800	6400	400	200
Ber	...	+	(+)	+	400	...	100	1600	1600	1600	800	200
Lag	...	(+)	+	...	100	+	+	400	400	1600	400	200
Lin	...	(+)	(+)	+	200	200	400	1600	400	800	200	100
Type IV—												
Hic	—	(+)	100	—	(+)	—	—	100	(+)	400	100	400
My	—	100	100	200	800	200	400	200	800	800	800	1600
Bow	—	+	200	200	800	400	400	200	800	400	800	1600
Gar	—	200	100	—	+	—	—	—	400	200	+	400
P 5	—	(+)	(+)	...	400	...	400	100	800	200	400	1600

+ =well-marked clumping at 1-100 but fluid still cloudy.

(+) =a slighter degree of agglutination.

— =no agglutination, or only a trace.

... =not tested.

\* Denotes that agglutination was not quite complete at that dilution and the lower dilutions.



Table I (b). *Agglutination tests on 19 Series I strains with sera prepared from Type II and Type IV strains.*

Designation of strains	Type II sera		Type IV sera				
	"Wat" titre 1-800	"Glid" titre 1-800	"Hic" titre 1-1600	"Bow" titre 1-1600	"Gar" titre 1-800	"My" titre 1-800	"My" titre 1-800
Type I—							
Litt	100	100	+	200	200	400	200
Cou	—	+	+	100	100	400	—
Win	—	—	—	+	100	200	—
Kn	—	(+)	(+)	+	100	200	—
Sher	—	+	+	100	200	400	+
Lis	—	+	+	100	100	200	—
Mit	—	+	+	100	200	400	(+)
Jor	—	—	—	+	+	(+)	—
Type III—							
Bunt	—	—	+	100	+	800	100
Mac	—	(+)	(+)	100	(+)	800	+
Type II—							
McP	100	200	100	400	100	800	100
Wat	800	400	100	100	100	400	—
Mi	800	800	200	400	200	800	400
Wy	800	800	100	400	+	800	200
Glid	800	800	400	400	200	800	100
Type IV—							
Hic	200	+	1600	800	400	400	800
Bow	400	400	1600	1600	400	1600	800
Gar	—	—	800	800	800	400	800
My	400	200	1600	800	400	800	800

**Type I sera.** Monovalent agglutinating sera were prepared from three of the 17 Type I strains, namely: "Cou," "All" and "Litt."

*Agglutination tests.* "Cou" serum (titre 1-800), the first prepared, was tested against the limited number of cocci then available and was found to agglutinate Type I strains only. The second serum was a high titre serum (1-1600) and agglutinated all Type I cocci (with one exception) in dilutions up to and beyond the titre of the serum for the homologous coccus. Most of the Type III and a few of the Type II and Type IV strains were also agglutinated, but only in dilutions of 1-100 and 1-200. The third serum agglutinated all Type I cocci (with one exception) up to the full titre of the serum for the homologous coccus, but this serum had a wider range of action on cocci of other types, particularly Type III, than the first two sera.

*Absorption tests.* Each of the sera was absorbed by some or all of the Type I strains and representative strains of other types. The homologous agglutinin was readily absorbed from each of two sera ("Cou" and "All") by all the Type I cocci and was not diminished, or only to a trifling extent, by cocci of the other types.

On the other hand the homologous agglutinin of the third serum was not

readily absorbed by Type I cocci. While the coccus producing the serum removed the whole of the agglutinin, other Type I strains were able to remove it only partially, *i.e.*, reduced the agglutinative power of the serum for the homologous coccus from 1-800 to at most 1-200, and after a second application of the cocci there was very little further reduction. Type III strains removed homologous agglutinin from this serum slightly, while a strain from each of the other types (II and IV) did not remove any; after a second absorption however there was slight reduction with a Type IV strain.

**SUMMARY.** With two of the three Type I sera the results were clear and definite. All the 17 Type I strains were agglutinated in higher dilutions of the sera than strains of other types, and all absorbed the specific agglutinin, which was not absorbed by other type strains.

The results with the third serum ("Litt") were less well defined and showed that the coccus producing the serum was not antigenically identical with the other two strains used to prepare sera.

**Type II sera.** Altogether six monovalent sera were prepared from Type II cocci.

*Agglutination tests.* The first two sera, made in the early part of the work with "Wat" and "Glid" strains, were tested against rather less than half the strains. The results with these sera are given in Table I (b). The two sera agglutinated the Type II strains at 1-400 and 1-800, except "McP" which was completely agglutinated only in 1-100 and 1-200 dilutions of the sera. Three Type IV cocci were agglutinated at 1-200 and 1-400, and one Type I coccus was agglutinated by both sera at 1-100. These sera, therefore, did not differentiate Type IV strains from Type II strains.

Subsequently monovalent sera were prepared from three more of Gordon's Type II strains and from one Type II strain<sup>1</sup> isolated in the early part of 1919 by Captain E. H. Shaw.

With these four sera there was a considerable amount of cross-agglutination, and no line of demarcation appeared between Type II and the other three types. Three of the sera each had a high titre for the homologous coccus, while the fourth failed to agglutinate the homologous coccus completely in dilutions higher than 1-100, though agglutinating other Type II strains well.

The Type II cocci exhibited a wide range of variation in the extent to which they were agglutinated by these four sera, and many strains of the other types were agglutinated in as high dilutions of the sera as Type II strains.

The most satisfactory serum for the grouping of the cocci by simple agglutination tests was that prepared from the "D" strain. On reference to Table I (a) it will be seen that this serum differentiated all excepting three

<sup>1</sup> This strain ("D") was subsequently identified at the Central Cerebro-spinal Fever Laboratory as a typical Type II strain; the results with the serum prepared from it are therefore included in Table I (a).



of the Type II cocci from other types by agglutinating them at the full or half titre of the serum.

*Absorption tests.* Absorption tests were carried out with five of the rabbit sera. Three of the sera ("Wat," "Glid" and "Off") were each absorbed with a small number of Type II strains, and only the coccus used to produce the serum was able to remove the homologous agglutinin completely. One strain ("McP") removed the homologous agglutinin from one serum ("Off") partially but failed to remove any from the other two sera. Other Type II cocci reduced the titre of each serum for the homologous coccus only slightly (to half titre at most). After a second addition of the cocci to two of the sera ("Wat" and "Glid") there was little or no further reduction with one serum ("Wat"), but with the other ("Glid") there was partial absorption in some cases. Strains of other types failed to absorb more than a trace of the homologous agglutinin.

With sera "Fos" and "D" a larger number of absorption tests were done. From one or other or both of these sera all the Type II cocci, with the exception of "McP" which absorbed only a trace from one serum, absorbed some of the agglutinin for the strain producing the serum, whereas representative strains from the other types did not remove any of it.

Even with these two sera all Type II strains did not exhibit an equal capacity for combining with the homologous agglutinin. While some strains absorbed as much as the coccus producing the serum, others were able to remove only half or quarter that amount; for example, the titre of the serum "D" was reduced from 1-1600 to 1-100 by the "D" coccus and some other strains, whereas other Type II strains reduced it only to 1-200 or 1-400.

Absorption tests were also carried out with a Type II goat serum. From this serum all but one (*i.e.* "McP") of the Type II strains tested absorbed the homologous agglutinin completely, strains from other types not removing any.

The strain "McP" mentioned above as having feeble capacity for absorbing Type II agglutinin from rabbit sera was also found to absorb only small amounts of agglutinin from this goat serum. Moreover, when a serum (goat) was prepared from this strain, it was found that Type II strains did not absorb its agglutinin. This strain, therefore, which perhaps at one time absorbed Type II agglutinin well, since it was one of the 32 cocci first investigated by Gordon, had acquired during artificial cultivation a high degree of individuality.

**SUMMARY.** Agglutination tests with monovalent sera prepared from six Type II strains did not clearly differentiate Type II from the other types. Not only was there pronounced cross-agglutination with these sera but also wide variation in the extent to which Type II strains were agglutinated by them.

Absorption tests on the other hand with two rabbit sera showed that Type II strains formed a distinct group, the members of which, however, differed among themselves in absorptive capacity. With a goat serum differentiation by absorption was clearer than with the two rabbit sera.

**Type III.** Agglutinating sera were prepared from three of the five Type III strains, namely "Mac," "Bunt" and "Mer": "Bunt" was Gordon's type strain<sup>1</sup> of the Type III sub-group; "Mac" was the strain which had been wrongly classified in the Type I sub-group.

*Simple agglutination tests.* "Bunt" serum agglutinated all the Type III strains tested in high dilutions of the serum; it also agglutinated the majority of the Type I strains and a few Type II and Type IV strains, but not so strongly as the Type III strains.

"Mac" serum agglutinated all the Type III strains at the full or half titre of the serum; it also agglutinated all Type I strains and several Type II and IV strains, but except in two instances at less than half the titre of the serum. The third serum ("Mer") gave very similar results.

*Absorption tests.* Each of the three sera was absorbed with all the Type III strains and the majority of the other strains. All the five Type III strains absorbed completely or nearly completely the homologous agglutinin from "Bunt" serum, while strains of other types absorbed little or none.

The same five strains which had exhausted "Bunt" serum also removed the homologous agglutinin completely or nearly completely from the other Type III sera, but a good many strains of the other types were found capable of removing agglutinin from these two sera.

From "Mac" serum four Type II, one Type I and two Type IV strains effected a partial removal of the agglutinin. The experiment with two of the Type II strains ("Mi" and "Wy") was repeated several times and on each occasion there was the same partial removal.

From "Mer" serum the two Type II strains ("Mi" and "Wy") and one Type IV strain ("My") also removed the homologous agglutinin partially, while two Type I strains and one Type II strain absorbed it slightly.

**SUMMARY.** With one Type III serum there was clear differentiation of Type III strains from other types both by agglutination and absorption tests.

With the other two sera there was not the same clear differentiation by agglutination and, although the Type III strains absorbed the homologous agglutinin from these sera as completely as from the first serum, strains of other types, particularly Type II, were found capable of removing appreciable amounts of their agglutinins. Thus agglutination and absorption tests with these sera showed that serological relationship exists between Type III and the other types.

**Type IV.** Four of the five Type IV strains were used to prepare agglutinating sera. The results of the agglutination tests are given in Table I (b). The results with the two Type II sera included in this Table have already been referred to.

<sup>1</sup> This strain when it had been three years in artificial cultivation was used again to produce a serum; this serum acted upon the great majority of the cocci tested, irrespective of type (see Table I (a)).



*Agglutination tests.* Each of the four strains produced a serum which agglutinated the Type IV strains to a higher degree than strains of any of the other types. There was some variation in the agglutinative power of the different sera on strains of Types I, II and III, two of the sera selecting mainly Type II strains, the other two agglutinating Type I and Type II strains indifferently. It will be noted also in Table I (b) that a particular strain was not agglutinated with equal readiness by the different sera.

The serum from one rabbit ("My") was tested at intervals during the course of immunisation. In Table I (b) the results with the first and last samples only, taken seven days after the third (30 mgrm.) and eighth (80 mgrm.) doses respectively, are given. The results with the different samples varied. The first sample had a wide range of action and agglutinated the majority of the strains of other types as well as, or even better than, the Type IV cocci. In subsequent samples the agglutinin for these other types diminished, and the final sample acted upon only a small number of strains, the titre of the serum for these being much lower than it had been previously.

One Type IV strain ("Hic") was used a second time to produce an agglutinating serum after it had been about four years in cultivation. The serum which was withdrawn three weeks after immunisation began had a wider range of action than the first serum produced with this coccus, the results, given in Table I (a), resembling closely those obtained with the first sample from the "My" rabbit.

*Absorption tests.* Each of the four sera was absorbed with the four Type IV strains as well as with representative strains of other types.

From "Hic" serum all the Type IV strains absorbed the homologous agglutinin completely or nearly completely.

Three of the four strains absorbed completely or nearly completely the agglutinins from two of the other sera, while the fourth ("Gar") removed it partially in both instances.

From the serum produced by this partial absorber ("Gar") none of the other Type IV strains was able to remove more than a slight amount of homologous agglutinin, and there was no further reduction even when a whole agar culture was used. Thirty-four other strains (Types I, II and III) were tested against this "Gar" serum with the result that some were found to remove slight amounts of the agglutinin while others failed to remove any or more than a trace. Similar results were obtained with thirty strains of Types I, II and III in absorption tests with the other Type IV sera.

The fifth Type IV strain (P 5) was tested with one serum only, *i.e.*, the second produced from "Hic," and was agglutinated by it in high dilution (see Table I a). This coccus also absorbed the homologous agglutinin of "Hic" serum.

**SUMMARY.** The above agglutination and absorption tests show that the Type IV strains form a group distinct from the other three types. All the strains absorbed the homologous agglutinin from a serum produced by one of them, and this agglutinin was not absorbed by strains of the other types. The members composing the group are, however, not completely identical, differing among themselves in agglutinogenic capacities.

## SUMMARY OF RESULTS WITH THE FIRST SERIES OF MENINGOCOCCI.

The serological reactions of forty-four strains of cerebro-spinal meningococci have been studied. These strains had been classified into four types at the Central Cerebro-spinal Fever Laboratory. The object of the investigation was to ascertain whether or not the classification would hold good if monovalent sera prepared in a different laboratory from members of each type were used.

*Simple agglutination tests.* Three Type I sera were prepared and two of these clearly differentiated the Type I strains from strains of other types. The third Type I serum agglutinated all the Type I cocci to full or half titre but this serum had more influence on strains of other types than the first two sera; *e.g.*, one Type III strain was agglutinated at half titre.

The sera prepared from Types II, III and IV were less specific and there was considerable cross-agglutination. One at least, however, out of each set differentiated fairly well; that is to say, the strains (or the majority of them in the case of Type II) of the same type as the homologous coccus were agglutinated in higher dilutions of the serum than strains of other types.

Type II strains showed less uniformity in agglutination with Type II sera than did Types III and IV strains in the presence of their respective type sera, several Type II strains agglutinating less well with the Type II sera than some strains of the other types.

*Absorption tests.* All the seventeen Type I strains absorbed the specific agglutinin completely from two Type I sera; strains of other types removed little or none of the agglutinin. From the third Type I serum Type I strains were able to remove the homologous agglutinin only partially and there was in addition slight absorption with Type III strains.

The five Type III strains absorbed the homologous agglutinin completely from three Type III sera. From one of these sera strains of other types absorbed little or none of the agglutinin, but with the two others there was definite cross-absorption, particularly with Type II strains.

All the Type II strains with one exception absorbed homologous agglutinin from two Type II rabbit sera. The amount absorbed however varied widely with the different strains, some absorbing as much as the coccus producing the serum, others only half or quarter that amount. From other Type II sera the coccus producing the serum was the only one found capable of removing the homologous agglutinin completely. Strains of Types I, III and IV did not absorb, except occasionally and in very small amount, homologous agglutinin from any of the Type II sera.

All the Type IV strains removed the homologous agglutinin completely or nearly completely from one Type IV serum while representative strains of other types failed to absorb any. One of the Type IV strains absorbed the homologous agglutinin only partially from two other Type IV sera and this



coccus produced a serum from which other Type IV strains were able to absorb only slight amounts of the homologous agglutinin.

With certain sera, therefore, prepared from members of each of the four types, a division of the meningococci into four distinct groups was effected by means of the absorption of agglutinin test. These groups correspond exactly to Gordon's four types.

With other sera from each type the differentiation into similar groups could not be made or was not so well defined, thus demonstrating that all the strains included in each type are not absolutely identical.

The following conclusions are to be drawn from my tests upon this series of meningococci:

1. Gordon's classification of the strains holds good if care is taken in the selection of strains for the production of type sera.

2. All the strains included in each type are not identical in their serological characters.

3. There is inter-relationship between the types, as shown by the results of cross-agglutination and absorption experiments.

## SECTION 2.

### Local Government Board strains.

This series contains thirty-one strains the serological characters of which have been reported on by F. Griffith in this *Journal* (1918).

Before proceeding to discuss my results with these strains it will be useful to summarise briefly the view expressed in the above report on the grouping of meningococci. F. Griffith has shown that the majority of spinal meningococci can be divided into two main groups by simple agglutination tests if selected sera are used, a few being relatively inagglutinable or agglutinated equally by sera of both groups. By the application of absorption tests it was found that the strains in each of the two main groups differed among themselves in absorptive capacity and could be divided into sub-groups. This division depends upon variations in the structure of their antigens. In Group I three different antigenic components were demonstrated which were designated "A," "B," and "C," "A" being the most complex, "C" the least. Group II was much more diverse and at least four different antigens were defined.

As mentioned on page 34 the strains in this series were to some extent selected. For example, having ascertained that strains with "C" antigen had no identical representatives among Gordon's types, I obtained the majority of the members of this sub-group of Group I. Many of the Group II strains were chosen on account of their poor absorptive capacity. A few strains could not be allotted definitely to either of the two groups.

The purpose of my investigation was to ascertain whether these spinal meningococci could be classified by means of sera prepared from Gordon's four types.

The great majority of the thirty-one strains were tested against eight sera, two from each of the four types; ten strains which were received late in the investigation were tested against five sera only. In Table II the results

Table II. *Agglutination and absorption tests upon the Local Government Board strains of cerebro-spinal meningococci with sera prepared from Gordon's four types.*

Designation of strains	Type I sera			Type III sera				Type II sera			Type IV sera			
	Aggl. with "Litt" serum 1-800	Aggl. with "Cou" serum 1-800	Absorp. of "Cou" serum	Aggl. with "Bunt" serum 1-3200	Absorp. of "Bunt" serum	Aggl. with "Mac" serum 1-1600	Absorp. of "Mac" serum	Aggl. with "Wat" serum 1-800	Aggl. with "Glid" serum 1-800	Absorp. of "Glid" serum (Goat)	Aggl. with "Hic" serum 1-1600	Aggl. with "Gar" serum 1-800	Absorp. of "Hic" serum	Absorp. of M 15 serum
M 4	800	800	N.C.	200	...	200 to 800	...	-	-	...	-	100	...	...
M 11	200	+	Nil	200	Nil	200	Nil	-	-	Nil	+	200	...	C.
M 12	400	100	Nil	1600	N.C.	1600	C.	-	-	...	100	+	...	...
M 14	400	+	Nil	400	Nil	100 to 800	Nil	+	-	Nil	+	200	...	C.
M 15	400	100	Nil	200	S.	200	S.	-	-	Nil	-	200	Nil	C.
M 16	400	-	Nil	100	Nil	100	Nil	+	+	...	100	200	Nil	...
M 17	400	+	Nil	1600	C.	1600	C.	-	-	...	-	-	Nil	N.C.
M 36	400	-	Nil	200	Nil	200	Nil	-	-	Nil	+	200	Nil	C.
M 40	400	+	P. (twice)	400	P.	400	S. & P.	-	-	...	-	-	...	S.
M 41	400	+	Nil	200	Nil	200	...	-	-	...	-	200	Nil	C.
M 43	400	200	Nil	1600	C.	1600	C.	-	-	...	+	-	...	C.
M 46	200	-	Nil	-	Nil	-	Nil	-	-	...	-	-	Nil	...
M 48	800	400	C.	200	Nil	200	Nil	-	-	...	-	100	...	...
M 67	...	400	C.	400	C.	400	S.	-	...	Nil*	-	...	...	S. & P.
M 68	...	400	N.C.	400	Nil	200	Nil	-	...	...	-	...	...	...
M 72	800	400	N.C.	200	V.S.	200	Nil	-	...	...	-	...	...	...
M 73	800	400	...	-	...	200	...	800	...	N.C.	-	...	...	...
M 77	100	+	Nil	400	Nil (twice)	100	...	100	...	Nil	-	...	Nil	V.S.
M 80	...	-	Nil	400	Nil	200	...	-	...	Nil*	-	...	V.S.	P.
M 18	+	...	...	-	...	...	...	400	200	C.	...	...	...	...
M 19	100	-	...	400	...	800	P.	1600	800	C.	100	+	Nil	...
M 20	100	-	...	200	...	800	S.	800	1600	C.	100	-	Nil	...
M 24	(+)	-	...	-	...	-	...	400	200	C.	-	-	Nil	...
M 31	+	-	...	-	...	-	Nil	100	200	Nil	100	+	Nil	Nil
M 32	-	-	...	-	...	-	...	-	+	Nil	-	-	Nil	Nil
M 33	+	-	...	+	...	200	...	200	+	...	800	400	N.C.	...
M 35	(+)	-	...	-	...	-	Nil	400	200	C.	-	-	Nil	...
M 82	...	-	...	-	...	...	...	400	...	N.C.	-	...	...	...
M 83	...	-	Nil	-	Nil	...	...	800	...	S.	+	...	Nil	Nil
M 86	...	-	Nil	-	Nil	...	...	+	...	Nil	-	...	Nil	Nil
M 89	...	-	Nil	-	Nil	...	...	-	...	Nil	200	...	N.C.	...

C. =absorption as complete as with the homologous coccus. N.C. =nearly complete.  
P. =partial. S. or V.S. =slight, or very slight. Nil =no absorption.

\* "Wat" rabbit serum.

of these tests are given. The designation of the strains is the same as in F. Griffith's report, M 4 down to M 80 belonging to his Group I, the remainder to his Group II.

*Agglutination tests.* One Type I serum ("Litt"), which had been shown in the tests on Series I to have a wider range of action than the other Type I sera, agglutinated all the Group I strains tested; this serum had little or no



action on Group II strains. The second Type I serum had a more limited range of action, agglutinating only nine of the nineteen Group I strains and leaving Group II entirely unaffected.

The two Type III sera agglutinated all the Group I strains (with one exception), three at the full titre of each serum. Three strains from Group II were also agglutinated by these sera, one serum agglutinating two strains in quite high dilution.

The two Type II sera agglutinated nine of the twelve Group II strains and two of the Group I strains, one of the two latter at the full titre of the serum. Other Group I strains were not acted upon.

Of the two Type IV sera one agglutinated five strains in Group II, one, M 33, at half titre of the serum, and two in Group I. The other agglutinated only one strain (*i.e.* M 33) in Group II and eight in Group I. It should be noted that M 33 was agglutinated well by both sera.

There is therefore a rough grouping by simple agglutination tests, Types I and III sera acting mainly on Group I strains, Type II sera mainly on Group II strains. There is evidence also of sub-grouping, the Type III and Type IV sera respectively picking out certain strains and agglutinating them in higher dilutions of the serum than other strains.

*Absorption tests.* One Type I serum ("Cou") was absorbed with all the Group I strains (except M 73) and three Group II strains.

Five Group I strains removed the homologous agglutinin completely or nearly completely, one removed it partially, while none of the others was able to absorb any of it.

The other Type I strain ("Litt") was absorbed with a few strains. M 48 absorbed the agglutinin partially, while M 11, M 16 and M 46 removed it very slightly; after a second addition of these cocci there was little or no further reduction.

The two Type III sera were absorbed with the majority of the Group I strains and a few Group II strains. Three strains from Group I, *i.e.* the three which had been strongly agglutinated by the sera, absorbed completely the specific Type III agglutinin from both sera; one Group I strain (M 40), which had removed Type I agglutinin partially, removed the homologous agglutinin partially from both Type III sera; a fifth strain (M 67), which had also absorbed Type I agglutinin, removed the homologous agglutinin completely from one Type III serum and slightly from the other. Of the Group II strains, one absorbed the agglutinin from one serum ("Mac") partially, and another Group II strain absorbed the same agglutinin slightly. A parallel result was obtained in the first series, several Type II strains absorbing the homologous agglutinin partially from this serum.

The Type II serum used systematically for absorption tests in this series was from a goat; this was the only Type II serum then available from which Type II strains were able to absorb homologous agglutinin completely. From this serum five Group II strains removed the homologous agglutinin com-

pletely and one (M 83) removed it partially. Of eight Group I strains used to absorb this goat serum, one, M 73 (see below), removed the homologous agglutinin nearly completely, while the others failed to remove any.

A few absorption tests were also done with the Type II rabbit sera used for agglutination tests. "Wat" serum was absorbed with three Group II strains, namely M 18, M 20 and M 24; these removed a small amount only of homologous agglutinin, and there was little further reduction on a second application of the cocci. "Glid" serum was absorbed with five Group II strains; M 19 and M 20 removed the agglutinin partially, whereas with M 18, M 24 and M 35 the absorption was very slight, even after a second addition of the cocci. Very similar results were obtained in the first series with these sera and Type II cocci.

One Type IV serum was absorbed with all the Group II strains and all those in Group I which had not yet been identified with any of Gordon's types. Two of the strains in Group II (M 33 and M 89) removed the homologous agglutinin from the serum nearly as completely as the coccus producing the serum; none of the other strains removed any of it even after a second absorption in some cases. The result with M 33 was confirmed with two other Type IV sera ("Bow" and "My").

After the completion of the above tests I noted in F. Griffith's report that the Group I strain, M 73, which had absorbed nearly completely the agglutinin from the Type II goat serum, had failed to absorb the homologous agglutinin of M 18 (a Group II coccus), but had removed partially the agglutinin of M 15, a Group I strain. I therefore re-tested this strain on a Type I serum, two Type II sera and one Type III serum. The strain removed homologous agglutinin nearly completely from the Type I and one Type II serum, and partially from the other Type II serum. From the Type III serum there was only a trace of absorption. Here then is an example of a strain which is capable of removing both Type I and Type II agglutinins.

The results of the agglutinin absorption test on this series of strains against sera made from Gordon's four types of meningococci are summarised as follows.

Table III. *Classification of thirty-one spinal strains of meningococci by means of the absorption test with sera prepared from Gordon's four types.*

Type I	Type III	Type II	Type IV	Indeterminate, absorbing 2 antigens	Unplaced	
M 4	M 12	M 18	M 33	M 40 (Types I and III)	M 11	M 16
M 48	M 17	M 19	M 89	M 67           ,,	M 14	M 31
M 68	M 43	M 20		M 73 (Types I and II)	M 15	M 32
M 72		M 24			M 36	M 46
		M 35			M 41	M 77
		M 82			M 80	M 86
		M 83				

The strains classified as Type I and Type III are Group I strains containing respectively F. Griffith's "A" and "B" agglutinin. Types II and IV correspond to sub-groups of Group II.



Table III shows that only sixteen of the thirty-one strains of cerebrospinal meningococci were completely identified with one or other of Gordon's four types; three strains were indeterminate, two having absorbed agglutinin from Types I and III sera and one from Types I and II sera, and twelve were not identified with any type by the absorption test. By the agglutination test of the latter twelve strains, nine (all Group I strains) were evidently related to Types I or III, two to Type II, and one was not agglutinated by any of the sera used.

The results of additional tests with the unplaced strains will now be considered.

Of the twelve strains which did not absorb agglutinin from any serum made with Gordon's strains F. Griffith had shown that six, all from Group I, were able to remove homologous agglutinin completely from a serum made with one of the strains (M 15) and therefore formed a sub-group. This agglutinin he designated "C" agglutinin of Group I. A little of this serum was kindly supplied to me and I tested it against all the unclassified strains and a good many other strains which had already been typed, with the result that five of the twelve unplaced strains were found to remove the agglutinin completely from this serum and one (M 80) to remove it partially, the other six having no effect. Several Type I and some Type III strains (including M 17 and M 43 in this series) absorbed the agglutinin from M 15 serum, in the majority of cases completely or nearly completely.

Except as regards M 80 my results with M 15 serum are identical with those obtained by F. Griffith.

With the other unplaced strains my results and his are also in close agreement.

The Group I strains M 16 and M 46 did not remove agglutinin from any of the four type sera; F. Griffith found that each of these strains was able to remove agglutinin only from its own serum.

With regard to the unplaced strains of Group II: M 31 was not agglutinated very well with the two selected sera but with other Type II sera it was agglutinated in high dilution. The strain absorbed a trace of agglutinin from one Type II serum but none from any other type serum. F. Griffith records that this strain absorbed agglutinin partially from sera of Group II, which appears to include Gordon's Types II and IV.

M 32 agglutinated with none of the sera in the table but agglutinated at 1-400 with another Type II serum. This strain did not absorb agglutinin from any of the type sera. F. Griffith found that it absorbed partially one of his Group II sera.

#### *Tests with Local Government Board sera on Gordon's four types.*

Orientation tests were also carried out with two sera supplied to me by Dr F. Griffith, one prepared from the Group I strain M 43, the other from the Group II strain M 18.

M 43 serum agglutinated Type I and Type III strains without distinction to the full or nearly to the full titre of the serum; there was no or only feeble action upon Type II and Type IV strains. The homologous agglutinin of this

serum was absorbed only by Type III strains. M 43 strain from which the above serum was prepared was originally a complex strain from which F. Griffith separated two daughter strains, one containing "A," the other "B" antigen. The strain of M 43 used in these absorption tests evidently contained only "B" antigen.

M 18 serum agglutinated all Type II and two Type IV strains in dilutions of 1-400 to 1-1600; two other Type IV strains and the Type I and Type III strains were but feebly acted upon or not at all.

Two Type II strains ("Wat" and "Keay") and M 18 itself absorbed completely the homologous agglutinin of this serum and two (a Type II and a Type IV strain) absorbed it partially while other Type II strains removed none or only traces. It is interesting to mention here that, although the above mentioned three strains showed equal capacity for removing the agglutinins from M 18 serum, two of them (M 18 and "Keay") were unable to remove the agglutinin produced by the third, *i.e.*, "Wat."

#### SUMMARY OF RESULTS WITH THE SECOND SERIES OF MENINGOCOCCI.

The general results of the investigation of this series of meningococci can be summarised under three heads.

(1) Sera prepared from Gordon's four types of meningococci do not suffice for the classification of all spinal meningococci. Out of thirty-one cerebro-spinal strains only sixteen could be completely identified with one or other of the four types; three were indeterminate and twelve were unplaced.

(2) F. Griffith's "A" and "B" agglutinins of his Group I are identical with Gordon's Type I and Type III respectively, and two of F. Griffith's four Group II agglutinins can be identified with Types II and IV.

(3) The twelve unplaced strains, not represented among Gordon's types, include a small group of strains related to Types I and III but not identical with either, and a residuum of highly individual strains.

As regards (1), the explanation why so many of the cerebro-spinal strains in this series could not be classified by absorption tests with Gordon's four type sera may be found in the fact that the source of material was not the same in the two series.

Gordon's strains were all obtained from military cases (*i.e.* adult cases) of cerebro-spinal fever, whereas the Local Government Board series was obtained from cases of meningitis occurring among the general population and included a large proportion from children.



## SECTION 3.

## Unselected strains.

This section deals with tests on eighteen strains, the serological characters of which had not been determined when they were sent to me. All the strains were derived from adult cases of cerebro-spinal fever.

I am indebted to Mr H. W. C. Vines for sixteen of the strains and to Dr W. M. Scott for two. These strains were investigated with the object of ascertaining what proportion could be identified with Gordon's four types.

Table IV gives the results of agglutination and absorption tests on these eighteen strains with six monovalent sera prepared from the four types, one each of Types I and IV and two each of Types II and III.

The results were clear and definite with fourteen strains which could be classified as follows:

Type I	Type II	Type III
1	9	4

Table IV. *Agglutination and absorption tests upon a series of unselected strains of cerebro-spinal meningococci.*

	Type I serum ("Cou" 1 in 800)		Type III sera				Type II sera		Type IV serum ("Hic" 1 in 1600)	
	Aggl.	Absorp.	("Bunt" 1 in 3200)		("Mac" 1 in 1600)		("Wat" 1 in 800)	("Gld" (Goat))	Aggl.	Absorp.
Smi	400	N.C.	400	V.S.	100	Nil	—	...	...	...
Cha	200	N.C.	400	P.	800	P.	—	...	—	...
Eld	400	N.C.	800	N.C.	800	N.C.	—	...	—	...
Bay	+	P.	200	V.S.	200	Nil	+	...	+	...
Sim	100	Nil	800	N.C.	800	N.C.	—	...	—	...
Bat	100	Nil	800	N.C.	800	N.C.	—	...	—	...
Am	+	Nil	400	P.	400	C.	—	...	+	...
Rou	—	Nil	400	N.C.	800	C.	—	...	+	Nil
Chid	—	...	—	...	...	...	1600	C.	+	...
Mitch	—	...	—	...	...	...	800	C.	—	...
Pow	—	...	—	...	...	...	800	C.	+	...
Mill	—	...	—	...	...	...	1600	C.	—	...
And	800	...	200	Nil	200	...	800	C.	200	Nil
Newb	—	...	—	...	...	...	1600	C.	100	...
Bay	—	...	—	...	...	...	800	C.	—	...
Mor	—	...	+	...	—	Nil	800	C.	+	Nil
Keay	—	Nil	—	...	—	Nil	400	C.	+	...
Ri	+	Nil	400	Nil	200	Nil	+	Nil	—	V.S.

The Type II serum used for absorption tests in this series was the goat serum from which, as has been recorded above, Type II strains readily removed the homologous agglutinin.

A few absorption tests were also carried out with two Type II rabbit sera ("Wat" and "Gl"); from these sera none of the strains (except the homologous coccus in each instance) was able to remove more than a slight amount of the homologous agglutinin at one absorption but after a second addition of the cocci one strain ("Mor") removed the whole of the agglutinin from both sera. It is interesting that this strain ("Mor") was able to combine with the agglutinins produced by two different strains ("Wat" and "Gl"), neither of which was able to exhaust the serum produced by the other ("Gl" or "Wat").

One of the Type III strains ("Rou") was atypical in agglutinogenic capacity. A rabbit was under treatment with this coccus a total period of two and a half months, the final dose being 80 mgrm., and during this period its serum was frequently tested. The last sample of serum failed to agglutinate the homologous coccus at 1-100 and only on one previous occasion did the titre rise to 1-100. The final sample of serum agglutinated chiefly Types I and III strains and these were equally acted upon but only in dilutions of 1-100 and 1-200.

The remaining four of the eighteen strains gave the following results: Two of the strains ("Cha" and "Eld") absorbed the homologous agglutinin from two type sera (I and III)<sup>1</sup>. A third strain ("Bay") although it was not agglutinated by the Type I serum appeared to absorb its agglutinin partially, an observation twice made. The fourth strain ("Ri") agglutinated best with the two Type III sera but failed to absorb agglutinin from either serum; there was also no absorption with this strain from five other sera, two Type I, two Type II and one Type IV.

With a view to studying the agglutinogenic capacity of this latter strain a serum was prepared from it. This serum, with a titre of 1-800, acted best on Type I and Type III strains, agglutinating the majority of them to the full titre of the serum; a good many Type II strains were also agglutinated but agglutination with one exception ("Glid" 1-800), was complete only at 1-100 or 1-200. Agglutinin absorption tests were carried out and the strain producing the serum was the only one which removed the homologous agglutinin completely. Type I and Type III, as well as cocci containing "C" agglutinin, removed it partially. There was very slight or no absorption from the serum with the Type II and Type IV cocci used.

#### SUMMARY OF RESULTS WITH THE THIRD SERIES OF MENINGOCOCCI.

Out of eighteen unselected strains fourteen could be identified with one or other of three of Gordon's four types. The remaining four were not typical; two were agglutinated best with Type III sera but absorbed agglutinin from both Type I and Type III sera; one gave a doubtful result, absorbing slightly from a Type I serum which had not agglutinated it; and one could not be identified by absorption tests with any type but was apparently related to Type III according to the agglutination test.

#### Tests with agglutinating sera from horses.

Altogether five horses were used for the purpose of producing agglutinating sera. Four of the animals were intravenously inoculated at five to seven days' intervals with gradually increasing doses of living meningococci. The initial dose in each case was 10 mgrm. and the largest dose given was 360 mgrm. The procedure in the fifth horse was a little different. This horse was immunised with Type IV cocci, and as an agglutinating serum for this type was then urgently required for trial a more intensive method was adopted. After three preliminary small doses during four days, the horse received the relatively

<sup>1</sup> Gordon (1915) has recorded a strain with similar combining capacities which he has termed "amphoteric."



large dose of 100 mgrm. on the sixth day and 200 mgrm. on the eleventh day, rising by stages to 300 on the twenty-eighth day. These doses were well borne but the serum could not, for the reasons stated below, be used for agglutination tests.

In this series of five horses no desensitising doses were given, and none of the horses died as the direct result of the injections. One, however (Mi 4, Type II), died twelve to fourteen hours after an injection of 300 mgrm. but the immediate cause of death was judged to be due to distension of the stomach following an over-feed of green food.

Samples of the sera were sent to Col. Gordon at frequent intervals, and when these gave satisfactory agglutination with the homologous cocci the horses were bled.

None of the sera, however, were used for the purpose for which they had been prepared, as it was found by Col. Gordon, after trial on a variety of strains, that they were not specific; that is, they did not differentiate the meningococci into the same types as rabbit sera. Some of the serum was used therapeutically in a few cases (one Type II and two or three Type III cases) apparently with good result, all the cases treated recovering.

For the purpose of comparing the relative specificity of different animal sera, the sera of these horses, as well as of others immunised later on for the production of therapeutic sera, were tested by me against a large number of strains of meningococci.

In Table V are given the results of agglutination tests with five horse sera and one donkey serum on thirty strains, all of which, with one exception (M 15), have been identified by means of rabbit sera with one or other of Gordon's four types.

Horse 1 was immunised with eight strains sent to me as representative of Type I. One of these strains ("Mac"), however, was subsequently found to be not a Type I but a Type III strain. It is not therefore surprising that there was no differentiation between Type I and Type III cocci, which were the only strains agglutinated well with this serum.

The donkey serum was produced with four typical Type I cocci and like Horse 1 serum divided the series into two groups, Types II and IV strains being unaffected, while Types I and III (except Bunting) were agglutinated in dilutions from 1-200 to 1-800.

The other Type I serum was a univalent serum with a high titre for the homologous coccus. This serum was one of the latest produced sera and some of the early strains were not then available for test. Many later ones were however included and altogether fifty-two strains were tested. Sixteen Type I strains were agglutinated in dilutions 1-1600 to 1-6400; thirteen Type III were complete at 1-400 to 1-800; seventeen Type II were complete at 1-400 to 1-800 and four Type IV 1-150 to 1-400. Thus, with this serum, while there was no sharp line of demarcation between Type I strains and the other types, all Type I cocci were agglutinated in higher dilutions than any other type.

Table V. *Agglutination tests with horse sera upon various strains of meningococci from the three series.*

Designation of strains		Type I sera			Type III	Type II	Type IV
		Horse 1	Donkey	Horse 35*	Horse 3	Horse 4	Horse 5
		Multivalent		Monovalent		Multivalent	
Type I—	Litt	1600	800	1600	—	800	200
	Cou	400	800	3200	—	400	+
	Win	400	800	6400	+	400	(+)
	Sher	1600	400	3200	+	200†	100
	Kna	400	400	3200	+ and 200	400	+
	Lis	800	800	...	—	400	(+)
	Mit	400	800	1600	100	400†	+
	Jor	1600	800	...	400	400	...
	M 4	800	800	...	+	800	(+)
	M 48	800	200	...	—	+	...
Type III—	Bunt	(+)	(+)	400	1600	1600	400
	Mac	400	200	800	1600	800	400
	M 12	800	200	...	1600	1600	400
	M 17	400	200	...	800	3200	100
	M 43	400	200	800	800	1600	400
“C”—	M 15	100	200	...	—	+	100
Type II—	McP	(+)	—	400	200	3200	200
	Wat	—	—	400	—	3200	100
	Mi	100	...	400	400	1600	400
	Wy	+	...	400	+	1600	+
	Glid	(+)	(+)	800	—	800	800
	Keay	—	—	...	—	800	+
	M 19	100	—	...	400	3200	800
	M 20	—	—	...	400†	1600	400
	M 24	—	—	...	—	800	+
Type IV—	M 33	—	—	...	(+)	800	800
	Hic	+	—	200	+	800	400
	Gar	200†	+	200	—	800	400
	Bow	100	+	400	200	1600	800
	My	+	(+)	100	200	1600	800

\* Horse 35 serum was tested on 34 strains additional to those in the table with the following results: 10 Type I agglutinated at 1-1600 to 1-6400; 10 Type III, 1-400 to 1-800; 12 Type II, 1-200 to 1-800; "Eld," 1-1600; "Ri," 1-200.

† Denotes that agglutination was not quite complete at that dilution and all lower dilutions.

Horse 3 was immunised with a single Type III strain. The serum agglutinated five Type III strains up to 1-800 and 1-1600 and agglutinated also a few strains from each of the other three types at lower dilutions.

Horse 4 was at first immunised with a single strain but, when it was found by Col. Gordon that the serum after six weeks' immunisation did not agglutinate all Type II cocci equally well, three other strains were added. With these four strains a serum of high titre was obtained, but it did not differentiate Type II from other types. Similarly the Type IV (multivalent) serum was not specific in its action, strains from other types being picked out irregularly



and agglutinated in as high dilution as the Type IV strains. The serum from this horse became useless after continued immunisation for another reason, namely, the occurrence of the phenomenon of pro-agglutination. Agglutination of the homologous and other cocci did not occur until the serum had been diluted 1-800 or more, and then only exceptionally was there complete agglutination of the cocci. The phenomenon of pro-agglutination was frequently observed with horse sera but usually it was evident only in the low dilutions of the sera.

Tests made with the sera of other horses confirmed as a rule those first obtained. Moderately specific sera were produced by Types I and III strains, while sera made with II and IV strains were generally, though not invariably, found to act well on strains of other types.

A few absorption tests were carried out. From Horse 4 serum (when monovalent) three Type II strains removed homologous agglutinin completely and four Type IV removed it partially.

Horse 35 (Type I) serum was absorbed with twenty-four strains. Four Type I and the "amphoteric" strain ("Eld") removed the homologous agglutinin completely, two Type III and one Type IV removed it partially, while the rest, including representatives of II, III and IV, removed only a slight amount or none at all.

From Horse 3 serum all the Type III cocci removed the homologous agglutinin completely, but some II and IV strains (*i.e.* those which combined with the homologous agglutinin in "Mac" rabbit serum) were also able to remove a good deal of it.

Agglutination and absorption tests with horse sera were in the main, therefore, in agreement with those obtained with rabbit sera.

#### **Tests with agglutinating sera from goats.**

Seven goats in all were immunised with three of Gordon's types of meningococci, three with Type I, three with Type II and one with Type III.

Goats were found to be very susceptible to the intravenous inoculation of meningococci. An initial dose of 1 mgrm. produced severe symptoms, and although the doses were very cautiously increased the animals were often unwell and refused their food twenty-four and more hours after an injection: they also lost weight. Three of the goats died after an injection two-and-a-half and four months after treatment began. None of these sera had attained a titre for the homologous coccus higher than 1-200. One goat died from causes unconnected with the experiment and in another case the experiment was discontinued.

Only two of the goats produced a serum with a satisfactory titre for the homologous coccus.

The serum of Goat 1 ("Litt") agglutinated the homologous coccus at 1-800 and other Type I cocci in dilutions 1-200 to 1-800. With the exception of one strain ("Gar," complete 1-100) no coccus belonging to other types was

acted upon at 1-100. This serum was absorbed with a selection of strains, and all the Type I cocci tested absorbed the homologous agglutinin, while none of the other types removed any.

The second satisfactory serum was made with a Type II coccus ("Glid"), which was agglutinated at 1-1600 after the goat had been about four months under treatment; other Type II cocci were agglutinated in dilutions 1-400 to 1-1600. The Type IV cocci and one Type I coccus were agglutinated at 1-100, while other cocci were unacted upon at that dilution. The serum of this goat proved very useful for absorption tests; all Type II cocci (with one or two exceptions) absorbed the specific agglutinin, whereas no coccus of other types was able to combine with it.

The two strains ("Litt" and "Glid") used for the immunisation of Goats 1 and 6 were also used to immunise rabbits. While from each of the goat sera the specific agglutinin was readily absorbed by the cocci of the same type as that used in producing the serum, from the rabbit sera specific agglutinin was removed completely by the homologous coccus only, other allied strains removing it only partially or slightly.

My conclusion from the above experiments is that goats are not suitable for the production of meningococcic agglutinating sera on account of their susceptibility and the tardy appearance of agglutinins. In the two cases where sera of good titre were obtained the agglutinins produced appeared to be specific both in agglutinative action and in relation to the absorption test.

#### **Variability of serological results.**

All who have worked with the meningococcus have noted irregularities in the results of serological tests.

The most common irregularity is variation in agglutinability of a strain. In the course of this investigation agglutination tests with the same serum and coccal suspension have often been repeated and the results have not always been identical, although the divergence has not been great. The widest variations between results on different occasions have been observed when different suspensions of the same strain have been used. This has been the case with Type II strains especially, but a similar observation has been made with a Type III and a Type IV strain. This particular irregularity has caused great trouble in absorption tests with Type II. On several occasions when it was necessary to use a fresh suspension of the coccus homologous with the serum, the new suspension has been found to agglutinate in very much lower dilution than the previous one. In one instance one suspension agglutinated at 1-3200 while a later, fresh, suspension was complete only at 1-100. Gordon (1917), Tulloch (1917), Scott (1917) relate similar examples.

In absorption tests there have been slight variations in the degree to which a particular coccus has removed homologous agglutinin from a serum on different occasions, but in general the results of repeated absorption tests have been remarkably constant.



It is sometimes the case that the strain used for producing the serum agglutinates at a less high titre than other strains of the same type. On two occasions, once with a Type II strain and once with a Type III strain, this feature was very marked. Elser and Huntoon (1909) express the view that inagglutinable but agglutinogenic strains are of frequent occurrence, but as Eastwood (1916) points out, their recorded laboratory data in support of this view are too scanty to justify such a generalisation.

#### **Varieties of meningococcus antigen.**

The results of the serological tests on the first series of meningococci indicate the existence, in this particular series of strains, of four distinct meningococcal antigens. Each of the four types of meningococci, into which Gordon has divided these strains, is distinguished by the predominance of one or other of these special antigens.

The different results obtained with sera made from strains belonging to the same type are attributable to variations in the amount and kind of the other antigenic components associated with the type antigen.

In certain strains one type of antigen greatly preponderates and hence it arises that sera made from such strains are very specific, picking out and agglutinating at high titre strains with the same antigen. If strains of this character are used for the preparation of sera for the classification of meningococci the division into types appears clear and definite. In other strains there are associated with the predominant antigen one or more components identical with or allied to the other type antigens. When sera made from such strains are employed the division of meningococci into types can no longer be maintained.

Of the different antigens, Type I appears to be more often present in a relatively pure state than the others and on this account it is easy to produce a specific Type I serum.

The strains of Type II show greater diversity of antigenic structure than those of Type I. There appears to be a variety of antigen common to all the Type II strains, but this is often associated with antigens related to the other types, as shown by the agglutination tests. There is, however, no uniformity in these associated antigens, which vary in different strains in kind and quantity. This is a possible explanation why Type II sera vary so much among themselves in range and degree of capacity to agglutinate meningococci of all types, and why the agglutinins of Type II sera are absorbed irregularly by Type II strains.

Type III antigen is commonly associated with antigens which agglutinogenically are related to Type I, but one strain produced a serum which was able to agglutinate strongly some Type II as well as Type I strains, and from which Type II strains absorbed the homologous agglutinin.

Type IV strains contain in common an antigen which is different from the other type antigens. This antigen is usually present with components which

produce agglutinins with combining affinities for Type II strains, but one of the Type IV strains showed definite evidence of serological relationship to Type I.

The four antigens described above are apparently not the only antigens which are common to groups of strains. Scott (1917) has distinguished by means of the absorption test at least eight distinct sub-groups in a series of 131 strains of meningococci (60 cerebro-spinal and 71 pharyngeal). F. Griffith (1917) has demonstrated three main antigens, designated "A," "B" and "C" (two of these, "A" and "B," are identical with Gordon's Type I and Type III respectively) in his Group I, and at least four in his Group II.

I have had experience with only one of these additional antigens, *i.e.*, the one producing F. Griffith's "C" agglutinin, the existence of which I have confirmed. This antigen is related to Types I and III but appears to be less complex than either, since the two latter can absorb the "C" agglutinin but the "C" antigen is unable to remove either Type I or Type III agglutinins.

#### SUMMARY AND CONCLUSIONS.

The serological reactions of three series (comprising ninety-three strains) of cerebro-spinal meningococci sent to me from three different laboratories have been studied.

The first series of forty-four strains came from the Central C.-S. F. Laboratory and were obtained from military cases of cerebro-spinal fever. They had already been classified into four types by means of Gordon's type sera. I prepared monovalent agglutinating sera from members of each type and with certain of these sera I was able by means of the absorption test to divide the strains into four classes corresponding to Gordon's four types. When, however, sera made from other members of each type were used the same differentiation of the strains could not be made, and in others was less well-defined.

The second series (thirty-one strains) came from the Local Government Board's Laboratory and were derived from cerebro-spinal fever cases occurring among the general population, including both adults and children. Simple agglutination tests with certain type sera made from Gordon's strains effected a division of the majority of the strains into two main groups, in agreement with F. Griffith's classification, a few strains being agglutinated by both sera, and a few not being placed on account of insufficient agglutination. With Type III and Type IV sera there was definite indication of sub-groups within the two main groups.

By application of the absorption of agglutinin test it was found that:

(1) Of the Group I strains four were identical with Type I and three with Type III (these strains were agglutinated to the full titre of the Type III sera); three absorbed agglutinin from more than one type serum, two absorbing the agglutinin from I and III and one from I and II; the remaining nine



could not be identified with any of Gordon's types. Six of these nine unplaced strains were shown by the use of a serum made from one of them to form a well-defined sub-group. This sub-group had already been defined by F. Griffith who designated the antigen present in these strains "C" antigen. Type I corresponded to strains of Group I containing his "A" antigen and Type III to his "B" antigen. Thus the "C" antigen of Group I which F. Griffith considers to be less complex than "A" or "B" was not represented in Gordon's military cases.

(2) Of the twelve Group II strains seven were identified with Gordon's Type II and two with Type IV. The remaining three could not be identified with any of Gordon's types.

The third series (eighteen strains) was derived from adult cases of cerebro-spinal fever. One of the strains was identified with Type I, nine with Type II and four with Type III. Of the remainder two absorbed agglutinin from Type I and Type III sera, one was indeterminate and one could not be identified by absorption tests with any of the four types.

It should be noted here that it was with an exceptional goat serum that the Type II strains in Series 2 and 3 were classified by absorption; none of the rabbit sera defined Type II strains so well as this goat serum.

My conclusions from the above results are as follows:

(1) If carefully selected type sera be taken as standards Gordon's four types can be well defined.

(2) These four types are not sufficient to include all meningococci which may be obtained from cases of cerebro-spinal fever.

(3) The proportion of strains which do not fall into any of Gordon's four types is greatest in cases of meningitis occurring among the general population.

(4) The division of the first series of meningococci into four types depends upon the existence of four chief antigenic components.

## PART II.

### THE PREPARATION OF ANTI-MENINGOCOCCUS SERUM.

The demonstration of the existence of at least four types of epidemic meningococci is of great practical importance from the point of view of the treatment of cerebro-spinal fever.

In the early years of the war cases of meningitis were treated with multi-valent serum from different sources. The results were disappointing and it was realised, in view of Gordon's findings, that one reason for this lack of success was that the serum used had not been prepared with strains which corresponded to the type of meningococcus infecting the patient.

In order therefore to ensure provision of more specific sera for the treatment of cases of cerebro-spinal fever in the Army, cultures of the four types of

meningococci were supplied by the Central Cerebro-spinal Fever Laboratory to the various makers of anti-meningococcus serum.

Clinical experience with these type sera during the epidemic of 1917 indicated considerable variation in the therapeutic value of different batches of anti-meningococcus serum, and it was recognised that experimental investigations were desirable for the purpose of defining the best methods of preparing and standardising anti-meningococcus serum.

Having already had some experience in the immunisation of horses, I was instructed by the Medical Research Committee to co-operate with Colonel Gordon in an attempt to improve the therapeutic value of anti-meningococcus serum.

Facilities in the way of accommodation for horses and of extra laboratory space were generously provided by the Committee of the Field Laboratories, University of Cambridge.

It was arranged that the testing of the serum was to be done by Colonel Gordon at the Central Cerebro-spinal Fever Laboratory and that no serum was to be issued for use which had not been approved by him.

*Preliminary Experiments.* The work began in the summer of 1917 when four horses were procured. These were used to test the relative values of the intravenous and intramuscular methods of injection, one pair being immunised with a Type I coccus, the other with a Type II coccus. Later on, other horses were obtained for the purpose of comparing the antigenic value of the living with that of the dead coccus.

Unfortunately these experiments were not carried far enough for definite conclusions to be drawn owing to the death or defect of one of the horses in each pair.

Horses were at that time very difficult to obtain and several were purchased which on account of age or infirmity were unable to stand the strain of immunisation with the meningococcus. Experience showed that only young healthy horses are suitable for this work. The horse is very susceptible to the meningococcus toxin, and after an injection almost invariably lies down. If the animal is aged or defective in wind or limb there comes a time, generally in the later stages of the immunisation, when the animal goes down and is unable to get up again unaided. My difficulties in this regard were removed in December, 1917, when arrangements were made with the Army Veterinary Department by which I was enabled to obtain horses which, while no longer fit for military duty, were suitable in every respect for serum production.

The intravenous and intramuscular comparisons were, however, carried sufficiently far to indicate that the latter method was inferior to the former in the production of agglutinins and opsonins, and, as the intramuscular injections produced large painful swellings which sometimes broke down, this method of immunisation was abandoned for the intravenous method. Intravenously inoculated animals were given gradually increasing doses at weekly intervals, according to the method employed at the Pasteur Institute, Paris<sup>1</sup>,

<sup>1</sup> I am indebted to Dr Dujardin-Beaumetz and M. Victor Frasey, Médecin Vétérinaire de l'Institut Pasteur, for demonstrating to me in December, 1915, the methods employed in the production of anti-meningococcus serum at the Pasteur Institute, Paris.



at first of killed culture, then of living culture; when the doses began to cause serious symptoms desensitising doses were administered two hours before the main dose as recommended by Dopter (1910).

The sera from these intravenously inoculated animals after nearly six months treatment on these lines were carefully tested at the Central C.-S. F. Laboratory for agglutinins, opsonins and anti-endotoxin.

Gordon's method of testing for anti-endotoxin was as follows: the growth from young cultures of virulent strains of meningococci were killed by ether, dried *in vacuo* and powdered; 0.1 gm. of this powder was carefully and thoroughly ground in an agate mortar and 5 c.c. of distilled water slowly added; the heavier particles were centrifuged out and an opaque watery extract was obtained of which 0.1 to 0.2 c.c. was lethal to mice inoculated intraperitoneally.

One minimal lethal dose of this extract and 0.5 c.c. of the serum were mixed together and incubated for 30 mins. at 37° C. The mixture was then inoculated intraperitoneally into a mouse. Control mice were inoculated with toxin and normal horse serum. With potent toxin the survival or death of the mouse was the criterion, but with weaker toxin the presence or absence of illness was regarded as a good index of the antitoxic value of the serum.

As evidence that a good serum must contain anti-endotoxin, Gordon (1918) records the following observation: he found that multivalent serum which gave the best results in military cases was differentiated from serum of less therapeutic value by ability to neutralise in 0.5 c.c. amounts one minimal lethal dose of the endotoxin of both Types I and II of the meningococcus.

In consequence of this observation it was decided to use, as far as was practicable, for the treatment of cases of the disease only those sera which contained a definite amount of anti-endotoxin.

The samples were reported to be excellent as regards agglutinins and opsonins but deficient in anti-endotoxin.

As the doses then reached were fairly high and had caused severe symptoms even after desensitisation, and as it appeared to me that further increase of dose to the level apparently necessary to make the serum protective might be fatal for some of the animals, I therefore decided to adopt the method of Amoss and Wollstein (1916) for the rapid preparation of anti-meningitis serum.

*Method of Amoss and Wollstein.* This method, which is, in principle, the intensive method of immunisation of Fornet and Müller (1910), was recommended by Amoss and Wollstein because it enabled them to prepare anti-meningitis serum within six to eight weeks and to give large doses of meningococci with safety.

The plan was to begin with small doses of living meningococci injected daily for three days followed by a period of rest of seven days, when another series of injections was made. After the first series the dose given on the first day of each subsequent series corresponded to that given on the last day of the preceding series. The temperature was taken hourly, beginning at the fourth hour, until it had reached its maximum and begun to decline. If the rise did not equal 2.5° C. to 3° C. the conclusion was drawn that the dose was too small. It was increased, therefore, for the injection twenty-four

hours later above the usual rate of increase. If, on the other hand, the temperature did not fall to normal within eighteen to twenty-four hours the conclusion was drawn that the dose given was too large. By following this plan doses could be regulated with nicety, and a maximum of reaction obtained with a minimum of danger. After the third or fourth series of injections a desensitising dose was given before the first injection in each series. The greatest reaction, as a rule, was that produced by the first injection, whereas the succeeding injections on the second and third days tended to produce less severe reactions. Hence the increase between the second and third injections might be larger than that between the first and second. The doses were measured by suspending the growth from one agar slant in 2 c.c. of physiological salt solution, giving definite amounts of this suspension beginning with 0.1 c.c. or one-twentieth of an agar slant. The largest amount of any single injection was one-fourth of each slant from seventeen different strains. Meningococci and para-meningococci were used alternately in the series of injections. When autolysate was also given this formed one series alternating with two series of living meningococci.

My method at first differed from the above only in the way the dose was estimated and in the use in the early stages of the immunisation of killed instead of living culture. The doses were weighed and in the first series were 5, 7.5 and 15 mgrm. or 5, 10 and 20 mgrm. according to the susceptibility of the horse. Each horse was immunised with one type of meningococcus only, the number of strains used varying from 1 to 6. Autolysates and sensitised cocci were administered, but not in any regular manner.

In series subsequent to the first the doses were increased very cautiously, and it was recognised later that the increase between the second and third doses was never so large as it might have been. Nevertheless, as reports from Colonel Gordon testified, the sera of the horses quickly showed a distinct advance, both in agglutinins and opsonins, on those produced by single weekly doses, and several also proved strong in anti-endotoxin. Eventually all the horses then under treatment yielded a serum which was declared to be sufficiently anti-endotoxic for use in cases of the disease.

My results, therefore, confirmed Amoss and Wollstein's statement that good anti-endotoxic serum can be produced by the method of three successive intravenous injections of meningococci at stated intervals.

For the successful routine use of the method, however, it appeared that considerable experience was necessary in the matter of dosage. If the dose, and particularly the third dose in each series, were not properly adjusted to reactive capacity, *i.e.* if it were insufficient or excessive, the serum of the horse instead of rising in titre would appear actually to fall.

No definite scheme of dosage can be laid down, for the doses will vary with the individual horse, but according to the method as practised here the following may be regarded as a normal course, the figures representing mgrms. of culture.



Series 1	2	3	4	5	6	7
5	15	30	50	90	140	150
7.5	20	35	60	125	200	225 to 250
15	30	50	100	150	300	350

Desensitising injections were given before the first dose in series 5, 6 and 7.

A horse which had received such a course of injections and had reacted typically to the third dose could be bled after the sixth or seventh series. This course could be shortened in a resistant animal by giving a larger dose at the end of the fifth series and substituting the seventh for the sixth. It will be noted that the first dose of the seventh series does not correspond with the last of the sixth. When the third dose exceeded 150 mgrm. (in this instance it was 300 mgrm.) it was of course impossible to begin the next series with that dose and increase in the same proportion as before, for then the animal would have been overwhelmed. It was found that good reactions were obtained with a dose slightly in excess of the preceding first dose, subsequent doses increasing, however, at a higher rate.

When the total weight of cocci given in the three days had reached 750 to 800 mgrm. the horse was allowed to rest for fourteen to twenty-one days, when the injections were begun again. After this period of rest horses were often found to be hyper-sensitive, and in order to avoid accidents it was necessary to give relatively small doses for the first series. Another plan, and one to be recommended when the horse had been some time under treatment, was to change the type of coccus. In such horses immunisation proceeds more rapidly than in new horses.

After having used the method for a period I found myself unable in one respect to confirm Amoss and Wollstein's statement, that the method is a safe one. In the later stages of immunisation the horse may develop hyper-sensitiveness after the first or second injections of a series, and then the cocci instead of producing a reaction are strongly toxic. One horse died after the second injection in a series, the dose being 200 mgrm.; another was gravely ill after this dose, but survived. Another horse died after the third injection (dose 300 mgrm.).

The symptoms in all these cases were the same. Two or three hours after the dose the horse begins to sweat; the temperature is only slightly raised above the normal; the respirations may be very greatly accelerated, and the animal lies prone; gradually the sweating extends over the whole body and becomes profuse; between the fourth and fifth hours the animal dies, or it may apparently recover for a time, and then die suddenly. These accidents can be avoided if the effects produced by the preceding injection are carefully studied. It is my experience that if after an injection the temperature rises only slightly and is still raised next morning it would be fatal to give another large injection. Also if the temperature rises very high, from  $41^{\circ}\text{C}$ . to  $42^{\circ}\text{C}$ ., and the general reaction is intense with some sweating, another injection on the next day is contra-indicated. In such cases it is advisable to intermit the

injections and begin again five or six days later, when the series may be given without danger.

*Modification of Amoss and Wollstein's Method.* The occurrence of fatalities and other considerations, however, had led me to try modifications of the method at the stage when the horse was liable to develop hyper-sensitiveness. It had frequently been observed in the later stages of immunisation that the third dose of a series often failed to excite a satisfactory reaction, and the possibility occurred to me that repeated large doses might in some cases be having the effect of neutralising anti-endotoxin already formed in the body instead of stimulating its further production. I began therefore as immunisation progressed to drop out one of the three doses and give only two, increasing the size of the second dose beyond that which I would have given had a third dose been contemplated. Then after one or two such series single large weekly doses were given which had been preceded in the afternoon of the previous day by a preparatory dose equivalent to about one-tenth the amount of the main dose. Thus, there was gradual concentration of dosage, and instead of three moderate reactions one single maximum reaction could be secured.

The plan was found to answer well and no horses were lost. Two series of doses are presented which were given to horses whose sera were passed for therapeutic use. Horse 43 was very sensitive and reacted well to every injection.

HORSE 43. TYPE II.		HORSE 35. TYPE I. (Changed from Type II.)	
Series I.		Series I.	
Nov. 25.	5 mgrm.	Jan. 30.	10 mgrm.
26.	5 „	31.	15 „
27.	10 „	Feb. 1.	30 „
Series II.		Series II.	
Dec. 5.	10 mgrm.	Feb. 10.	30 mgrm.
6.	15 „	11.	40 „
7.	25 „	12.	80 „
Series III.		Series III.	
Dec. 16.	25 mgrm.	Feb. 20.	75 mgrm.
17.	30 „	21.	100 „
18.	50 „	22.	150 „
Series IV.		Series IV.	
Dec. 27.	50 mgrm.	Mar. 3.	15 mgrm. and 2 hours later
28.	60 „ Very strong reaction, 3rd dose therefore not given		135 „
		4.	200 „
Series V.		Series V.	
Jan. 6.	60 mgrm.	Mar. 11.	35 mgrm. at 4 p.m.
7.	80 „	12.	350 „ 11 a.m.
8.	110 „		
Series VI.		Series VI.	
Jan. 17.	10 mgrm. and 2 hours later	Mar. 23.	50 mgrm. at 4 p.m.
	100 „	24.	550 „ 11 a.m.
18.	150 „		Reaction not satisfactory



## HORSE 43. TYPE II.

## Series VII.

Jan. 27. 25 mgrm. at 4 p.m.  
 28. 250 „ 11 a.m.

## Series VIII.

Feb. 6. 45 mgrm. at 4 p.m.  
 7. 450 „ 11 a.m., strong  
 reaction  
 12. Serum sample weakly anti-endotoxic  
 14. Bled } serum strongly anti-endotoxic  
 15. Bled )  
 17. Serum sample weakly anti-endotoxic

## Series IX.

Feb. 24. 50 mgrm. at 4 p.m.  
 25. 550 „ 11 a.m.

Reaction identical with that following Series VIII  
 Serum on 7th and 8th days not approved.

HORSE 35. TYPE I.  
 (Changed from Type II.)

## Series VII.

April 7. 50 mgrm. at 4 p.m.  
 8. 600 „ 11 a.m.

Serum approved on the 3rd, 5th and 6th  
 days, but not on the 7th, 9th and  
 12th days.

Horses were bled after the 450 mgrm. and again after the 550 or 600 mgrm. dose, if the reactions had been satisfactory. A good febrile and general reaction after the dose preceding the bleeding of the horse was regarded as important and a few typical temperature reactions are reproduced (Charts I and II). The first curves are after a three-series and a two-series course of injections; the other two followed single injections.

During the course of the work many attempts were made to determine what interval of time should follow an injection before bleeding a horse. It was hoped that information on this point might be obtained by taking samples of the serum at frequent intervals after an injection and sending them to be tested by Colonel Gordon. If the reports on the early samples were favourable the horse was bled.

The results were very conflicting and appeared to indicate a good deal of individual variation. Some samples were reported as protective on the first, second or third days, and when the horse was bled five to seven days after the injection, the serum was found not to be protective. Other sera were not protective during the first few days but were reported as being so later on, fifth to eighth days. Another horse would yield a protective serum on every occasion on which it was tested during the first eight days.

The observations made, therefore, do not enable me to state what is the best day for bleeding a horse after an injection. Horses were bled as a rule on the seventh or eighth days, and these are probably the most suitable intervals.

The evidence was also conflicting in regard to the stage of immunisation when a protective serum might be expected. As the sera which were passed almost invariably came from horses which had reacted strongly it was hoped that intensity of reaction might be used as an index when to bleed. But it was found that of two horses, comparable in respect to dosage, reaction and interval after injection, the serum of one would be approved, while that of the other would be rejected. Also a horse which had yielded a protective

serum on one occasion would on the next, after a larger dose, yield a serum without protective property, although the reactions to the inoculations on both occasions had been identical.

The explanation of these irregular results may perhaps be found in individual variation in the time and duration of maximum production of

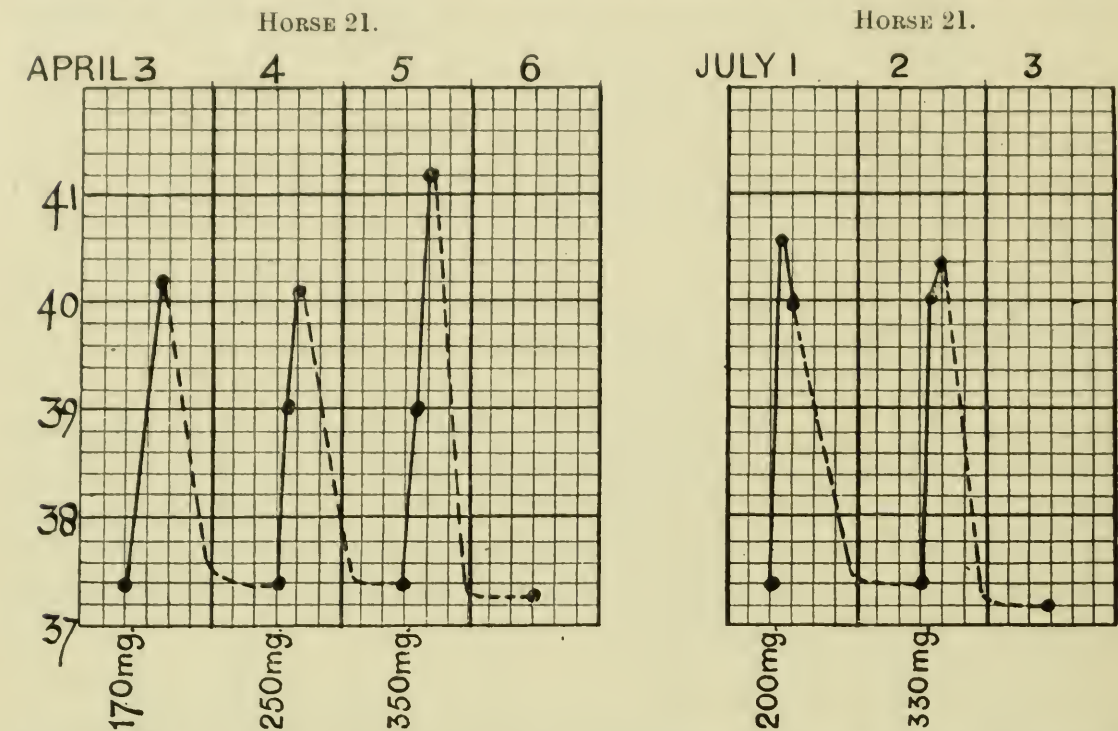


Chart I. Two hours before the first dose in each of these two series of injections a desensitising dose of 15 and 20 mgrm. respectively was given.

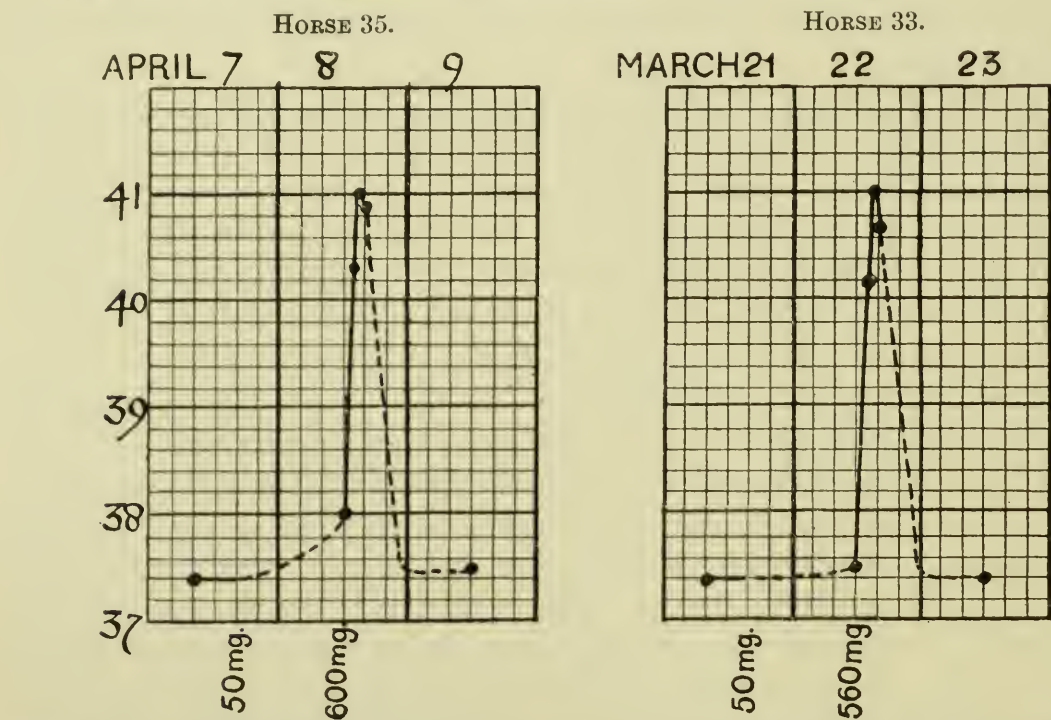


Chart II. Each vertical space in the charts represents an interval of six hours. The thick vertical lines mark off the days.



anti-endotoxin. In some immunised horses the curve of anti-endotoxin may be steeply-shaped, rising to its maximum on different days in different horses, and this may be the result when the immunising dose is insufficient to provoke a strong reaction. In such cases it would be a matter of chance whether the horse was bled on the right day or not. In other horses the anti-endotoxin curve is broad and protective sera may be obtained on several occasions from such animals. On the other hand the test for anti-endotoxin may not always give consistent results. Some experiments are described in a subsequent section which show that the mouse varies greatly in its susceptibility to the meningococcus toxin.

For these reasons I have not drawn any final conclusions as to what is the best method of injecting horses for the production of anti-meningococcus serum. While I have produced sera by two methods which have given satisfactory clinical results, the modification of the method of Amoss and Wollstein which I have described (p. 63) appears to me to have some advantages over the original from the point of view of safety as well as for technical reasons.

*Immunisation of horses with dried cocci.*

At Colonel Gordon's request I immunised four horses with dried cocci, two with Type I and two with Type II, according to a scheme of dosage proposed by him.

The horses were under treatment for a period of about three months and were given three doses weekly. The initial dose was 0.5 mgrm. and the final 12 mgrm., each dose being repeated before the next higher dose was administered. The dried cocci had no bad effect upon the horses and the reactions were for the most part very mild.

Samples of the serum were sent to Colonel Gordon to be tested. No anti-endotoxin was demonstrated on any occasion in the Type II horses. The sera of the Type I horses were reported protective on the first and third days after the 8.5 mgrm. doses, and one was protective on the third day after the 11.0 mgrm. dose, while the serum of the other was not protective after the 12.0 mgrm. dose.

The serum of the first of the Type I horses was put up for trial and comparison with the sera produced by the injection of the living coccus.

**Tests of the potency of anti-meningococcus serum.**

As stated above the routine testing of the sera of the horses was done at the Central Cerebro-spinal Fever Laboratory<sup>1</sup>.

This plan was adopted not only because it was impossible for me, working single-handed as I was, to carry out in addition to the detailed work of producing the serum the very numerous tests which were requisite in the investigation, but also because the method of test was then in the experimental stage.

<sup>1</sup> I wish here to thank Colonel Gordon for the promptness with which he reported on the large number of samples submitted to him.

Subsequently, when the test had been more or less standardised, I was able to do a few tests for which Colonel Gordon kindly supplied me with some dried cocci. The sera chosen for testing were those which had already been passed by Colonel Gordon.

The mode of preparation of the toxin was as described in the *British Medical Journal* (*loc. cit.*) except that an ordinary instead of an agate mortar was used to grind up the cocci and the toxin and serum were incubated for half-an-hour in small test tubes instead of in watch glasses.

In a preliminary experiment the toxicity of the Type II toxin was tested. Two mice each received 0.1 mgrm. and two mice each received 0.2 mgrm. of the toxin. All remained well.

In the next experiment the same toxin was used but the dose was increased to 0.25 mgrm. Of two control mice, one died in thirty-six hours, the other survived. Two mice to which toxin + anti-meningococcus serum had been administered remained well, while a third died within twenty-four hours.

In a third experiment fresh Type II toxin was used, and the dose was 0.2 mgrm. The two controls died within forty-eight hours, while of four mice receiving toxin + anti-meningococcus serum three died and one remained unaffected. In a fourth experiment with Type II toxin the dose was 0.15 mgrm. (two controls received this dose and a third received 0.2 mgrm.). The three control mice lived and of eight receiving anti-meningococcus serum + 0.15 mgrm. of toxin seven remained well and one died.

The next three experiments were with Type I toxin. In the first toxin only was injected, four mice receiving 0.1 mgrm. and five mice 0.15 mgrm. Of the four two died and two survived; of the five three died and two survived.

In the next experiment there were six controls, two of which received 0.1 mgrm., two 0.15 mgrm. and two 0.2 mgrm. The second pair and one out of each of the other pairs survived, the remaining two dying within twenty-four hours. Three mice received toxin + anti-meningococcus serum, the dose of toxin for each mouse being 0.2 mgrm.; of these two survived and one died, *i.e.*, there was exactly the same proportion of survivors among the serum treated mice as among the controls. In a third experiment with Type I toxin three samples of anti-meningococcus serum from the same horse, taken on different days after an injection, were tested; one of these sera had failed to pass Colonel Gordon's test. For each sample three mice were used; there were three controls; the dose of toxin for each mouse was 0.15 mgrm. Of the three controls two were dead on the next day and one survived. The same result was obtained with one serum sample. From each of the other two sets (including the one inoculated with the rejected serum) one mouse was dead within two days and the other two survived.

It is interesting to point out that, after eliminating Experiment 4 where the dose of toxin was insufficient, the percentage of mice which survived the inoculation of toxin and normal serum is practically identical with that for mice receiving toxin and anti-meningococcus serum.



Two experiments were carried out with living meningococci from eighteen-hour old cultures on glucose agar. In the first experiment four mice were used, and the dose was 10 mgrm. of a Type II coccus. The two controls remained well, while the two mice receiving anti-meningococcus serum died within twenty-four hours.

The second experiment was with a Type I coccus and the dose for each mouse was 15 mgrm. There were eight controls and eight inoculated with Type I serum. Of the eight controls seven died within two days and one survived. Of the eight serum treated mice five died within three days and three survived. Here the evidence was in favour of the presence of protective bodies in the serum. But if the figures of the two experiments with living cocci are combined it is found that, as in the experiments with endotoxin, the percentage of mice which survived the injection of culture only is the same as that for the serum treated mice.

The results of these tests, therefore, are not in agreement with those obtained by Colonel Gordon with the same sera, but the experiments are too few in number from which to draw final conclusions as to the value of the test for estimating the anti-endotoxic potency of anti-meningococcus serum.

#### REFERENCES.

- AMOSS and WOLLSTEIN (1916). *Journ. Exper. Med.* XXIII. 403.  
DOPTER and BIOT (1910). *Comptes Rendus Soc. Biol.* LXIX.  
EASTWOOD, A. (1916). *Reports to the Local Government Board on Public Health and Medical Subjects*, N.S. No. 110, p. 35.  
ELSER and HUNTOON (1909). *Journ. Med. Research*, xx.  
FORNET and MÜLLER (1910). *Zeitschr. f. Hyg.* LXVI. 215.  
GORDON, M. H. and MURRAY, E. G. (1915). *Journ. Roy. Army Med. Corps.* xxv.  
GORDON, M. H. (1917). *Medical Research Committee, Special Report Series*, No. 3.  
GORDON (1918). *Brit. Med. Journ.* Sept. 28.  
GRIFFITH, F. (1917). *Reports to the Local Government Board on Public Health and Medical Subjects*, N.S. No. 114 and (1918) *Journ. of Hygiene*, xvii. Nos. 2 and 3.  
SCOTT, W. M. (1917 and 1918). *Reports to Local Government Board on Public Health and Medical Subjects*.  
TULLOCH, W. J. (1917). *Journ. Roy. Army Med. Corps.*

## CARRIAGE OF COLIFORM BACILLI BY THE ORIENTAL HORNET (*VESPA ORIENTALIS* FABR.).

BY P. A. BUXTON, M.A., F.E.S., M.R.C.S.

*Fellow of Trinity College, Cambridge.*

THE large red Oriental Hornet (*Vespa orientalis* Fabr.) must be extremely familiar to all who served in Mesopotamia. The Rev. F. D. Morice, who has been good enough to identify my specimens, tells me that it can barely be distinguished from *Vespa crabro* L., the European hornet, except by its colour. It is widely distributed in the Mediterranean countries up to lat. 47° N. and occurs S. to Abyssinia and Madagascar, E. to India, and also as an introduced species in certain parts of S. America.

In Mesopotamia the nests are made in hollows in mud walls, under old roofs, in the brick sides of wells and similar situations. The hybernated females appear in the early spring, and workers are first seen in mid-June. During August, September and October they are exceedingly abundant and sometimes conceal from view the joints of meat on which they are feeding in the butchers' shops. They also devour all sorts of fruit, particularly fresh dates in the bazaars. It is a matter of common knowledge that this insect feeds on all forms of household refuse, especially faeces of men and animals, and the dried carcasses that one finds so frequently on the edge of Arab towns. I once observed hundreds of workers gorging themselves at a stream of blood and putrid offal which oozed from beneath the door of the Jewish slaughter house in Baghdad; this was in September 1917.

Graham Smith (1916, p. 504) has recorded visits of common wasps to traps baited with human faeces in Cambridge. I know of no other reference to this habit of the wasp.

During the summer and autumn of 1918, while I was serving in the Central Laboratory, Amara, Mesopotamia, I devoted a little time to examining the bacteria carried by this foul-feeder. I microscopically examined the contents of the gut of 58 workers, and prepared plate cultures from 64 on MacConkey's bile salt-litmus-lactose medium, with the following results.

*Dissections.* I began the series of dissections expecting to find cysts of intestinal protozoa, and eggs of intestinal worms, just as one finds them in the house-fly. My method was to examine the gut contents after treatment with iodine. It soon became apparent that the digestive processes of the hornet are rapid and complete. If a hornet is fed experimentally on human faeces, and dissected an hour after feeding, nothing recognizable as faeces can be found in the gut. The 58 wild-caught hornets I examined were from



slaughter houses and butchers' shops, dumps of household refuse, fruit stalls, and the sides of the porous clay water-coolers known in Arabic as "hab." The hornets were killed at once but 34 of them contained no solid matter of any sort in the gut, 20 contained débris and four "apparent faeces." I have already discussed the points which lead one to conclude that any given material in the gut of the house-fly is human faeces (see Buxton, 1920). I found no eggs of parasitic worms or cysts of protozoa in the "apparent faeces" on these four hornets. One of the four was eating dates in a fruit stall, the three others on different days drinking water from the outside of a water cooler in the billet of the Assistant Director of Medical Services. The material recorded as débris consisted for the most part of fine grit, which I presumed to be dust, accidentally ingested with the food. The result of the dissections was therefore as follows:

No. of workers dissected ... ..	58
No. of guts containing no solid matter ... ..	34
No. of guts containing débris, mostly mineral ... ..	20
No. of guts containing "apparent faeces" ... ..	4

*Platings.* The rapidity of the hornets' digestive processes seemed to indicate that any results obtained from dissection would be misleading, because only a few of these workers which had been feeding on faeces would be found to contain material which could be recognized microscopically as faeces. The series of workers whose organs were plated shows that this was the case and that the hornets were much more frequently infected with intestinal bacteria than one would have guessed from the dissections alone. Hornets for plating were collected in a sterile tube and carried in a wet towel to the laboratory in order to keep them cool; they always arrived alive. As is very well known (Buxton, 1914; Latter, 1913), Hymenoptera die quickly in hot weather if they are confined in a tube or small box. The insects were dissected with aseptic precautions. In the case of the first dozen insects the mouth-parts, legs, wings and various parts of the gut were plated separately on MacConkey's medium. I found that if a hornet was carrying coliform organisms at all, I could generally recover them from all parts of the body within and without. Subsequently I merely broke up the insects in sterile broth and plated a loopful of the fluid on MacConkey's medium. A very great number of species of bacteria frequently grew in spite of the bile-salts present in the medium. Agar subcultures of suspicious colonies were tested with such sugars as were available, and the motility of the organism and its reaction to litmus milk and Gram's stain noted.

The Table (p. 70) shows the coliform bacilli in three groups. Group *a*, lactose-fractors of uncommon types, each of which was isolated once only, the first two from the same hornet. I do not regard the recovery of these bacteria as evidence of faecal contamination. Group *b*, common lactose-fractors. Each species was found a number of times, often two species in one hornet. Of 64 hornets plated 23 were found to be infected with one or more of these

common intestinal organisms, which were generally present in immense numbers when present at all. I tested more than half the bacilli in this group not only with the five sugars mentioned in the Table but also with dextrin, maltose, salicin, litmus milk and the Voges Proskauer reaction. All those tested agreed in every particular with the well-known inhabitants of the intestine with which I have identified them. Group *c*, an organism which gave the cultural reactions of Shiga's bacillus, was found on three of the 64 hornets: from one of these individuals I also isolated what appeared to be Morgan's No. 1 bacillus, and a fourth hornet yielded Morgan's bacillus without the Shiga-like organism. These non-fermenters of lactose occurred

Table showing various species of coliform bacteria isolated from Oriental Hornets at Amara, Mesopotamia, in the summer of 1918.

Group	Number of occurrences	Litmus broth					Motility	Name	
		Lactose	Glucose	Dulcitate	Mannite	Saccharose			
<i>a</i>	1	AG	AG	O	O	AG	?	?	*
	1	AG	AG	O	AG	O	+	<i>B. gr�n্থali</i> *	
	1	AG	AG	O	AG	AG	+	<i>B. cloacae</i> *	
<i>b</i>	6	AG	AG	AG	AG	O	+	<i>B. coli communis</i>	
	8	AG	AG	AG	AG	AG	+	<i>B. coli communior</i>	
	11	AG	AG	O	AG	AG	−	<i>B. lactis aerogenes</i>	
	3	AG	AG	O	AG	O	−	<i>B. acidi lactici</i>	
<i>c</i>	3	O	A	O	O	O	−	<i>B. dysenteriae</i> (Shiga)	
	2	O	AG	O	O	O	+	Morgan's No. 1 bacillus	

\* These three organisms were not investigated in detail. The last two agree with *B. gr n্থali* and *B. cloacae* as far as sugars are concerned. They may or may not have been derived from faeces; for the purpose of the present paper, which proves that *Vespa orientalis* is a carrier of faecal organisms, it is immaterial whether they were or were not!

sparingly among rich growths of one or other of the organisms of Group *b*. Capt. G. Shanks, I.M.S., was so kind as to test the three organisms which had the cultural characters of Shiga's bacillus, with specific agglutinating serum: none of them were agglutinated except at insignificantly low dilutions. We had no specific serum with which to test Morgan's bacillus on the two occasions on which we isolated it. In spite of this I think these platings give us undeniable proof of the carriage of bacteria derived from faeces by these hornets. Had I only found a single type of coliform bacillus giving, let us say the familiar sugar reactions of *B. coli communis*, it might easily have been explained by supposing that these hornets normally harboured a "coli-like" organism which was not faecal in origin, but could not be distinguished from *B. c. communis* by any of the tests applied. Such an explanation does not meet the case we are considering. In these hornets which are known devourers of human faeces, four types of bacilli are commonly found: these agree in every criterion which can be applied with the common faecal organisms *Bacillus coli communis*, *B. c. communior*, *B. lactis aerogenes* and *B. acidi lactici*: among these lactose-fractors we find much more rarely organisms resembling Shiga's bacillus and Morgan's No. 1 bacillus, and these



organisms never occur except along with the lactose-fermenters. We may therefore safely assume that we have been correct in our identification of these bacilli, and that at any rate the majority of them were derived from faeces.

The relative frequency of the organisms in Group *b* attracted my attention. As previously stated I isolated *B. coli communis* six times, *B. c. communior* eight times, *B. lactis aerogenes* eleven times, and *B. acidi lactici* thrice. The commonness of *B. lactis aerogenes* may be due to its being able to withstand some unfavourable condition in or on the hornet for a longer period than the other types can. It was suggested to me that the sugar reactions of these bacilli might change while they are in the hornet's gut: I know no shadow of evidence which might support this view. I subcultured a number of these Group *b* organisms daily for 15–20 days, but their sugar reactions remained unchanged.

The result of the platings was therefore as follows:

No. of hornet workers examined	...	...	...	...	64
No. from which coliforms were isolated	...	...	...	...	23
No. from which pathogenic coliforms were isolated	...	...	...	...	4

(viz., Shiga twice, Morgan once, Shiga and Morgan once).

*Conclusion.* The Oriental Hornet (*Vespa orientalis* Fabr.) frequents piles of domestic refuse and human faeces, also fruit shops. It appears to digest what it eats rapidly, so that microscopic examination of the gut contents does not give us a correct idea of the very high degree to which it is contaminated. Sixty-four workers were plated; of these twenty-three were carrying well-known types of intestinal bacteria. I state that these bacteria were faecal, not that they *resembled* well-known faecal organisms for these reasons: they agreed in every test with the species with which I have identified them; the lactose-fermenters were common, and those which did not ferment lactose were rarer, and occurring always among the lactose-fermenters.

On the whole I concluded that the individual hornet is probably nearly as heavily infected with pathogenic coliform organisms as the individual house-fly; it is however not so exceedingly common as that insect, and it rarely enters mess-rooms and kitchens. As a spreader of infectious disorders of the bowel it is probably of very slight practical importance. It will be time enough to open a campaign against the hornet when the spring and autumn fly-plague in the Arab towns is a thing of the past.

#### REFERENCES.

- BUXTON, P. A. (1914). Notes on Tunisian and Algerian Insects. *Entomologist's Record*, xxvi. 66.  
 — (31. I. 1920). The Importance of the House-Fly as a Carrier of *E. histolytica*. *Brit. Med. Journ.* i. 142.  
 GRAHAM-SMITH, G. S. (1916). Observations on the Habits and Parasites of Common Flies. *Parasitology*, viii. 440.  
 LATTEr, O. H. (1913). *Bees and Wasps*. Cambridge Manuals of Science and Literature.

## ON DEFENSE RUPTURE AND THE ANTAGONISTIC ACTION OF SALTS

BY C. SHEARER, F.R.S.

IN a recent number of this *Journal*<sup>1</sup>, I have pointed out that the well-known antagonistic action of Ca to Na, was probably at the bottom of the peculiar "defense rupture" action of Ca, as described by Bullock and Cramer<sup>2</sup>, in relation to infection with the organisms of gas gangrene.

In the preceding number of this *Journal*, Cramer and Gye<sup>3</sup> give some additional experiments, which they claim demonstrate that the matter is not so straightforward and simple.

In these new experiments it is shown that if the gas gangrene organisms or their spores are washed in Ringer's solution instead of NaCl, they are unable to kill the mice into which they are injected. Obviously here it is not a question of the unbalanced action of the Na playing a part in the question.

It is now clear from these experiments and also from other results obtained by Cramer and Gye, published since the appearance of my original paper, that these authors are dealing with a much more complicated series of events than at first seemed apparent. Two widely different salt actions enter into the problem.

First, the antagonistic action of Ca-ions to the Na-ions on the bacteria, such as I have described in my paper. Secondly, a defense rupture action as these authors call it, of the Ca-ions and numerous other poisonous substances on the tissues of the animals, in the absence of which the gas gangrene organisms are wholly unable to set up infection in the animals into which they are inoculated.

If the emulsions of gas gangrene organisms employed are free from spores, the effect will be as I have described in my experiments with *B. anthracis*. The bacteria will be killed by the NaCl or their powers of resistance so lowered that the leucocytes soon get rid of them when they are injected into the animal. If a little Ca is added to the emulsion just before injection, and the action of the NaCl has not been too prolonged, the bacteria will recover their normal condition, and as the Ca also devitalizes the tissues of the animal at the point of injection, infection will occur. On the other hand if the action of the NaCl

<sup>1</sup> Shearer (1919), *Journ. Hyg.* XVIII. 337.

<sup>2</sup> Bullock and Cramer (1919), *Proc. Roy. Soc. Ser. B.* xc. 513.

<sup>3</sup> Cramer and Gye (1920), *Journ. Hyg.* XVIII. 463.



on the bacteria has been too prolonged, then the addition of the Ca will not produce infection, as the bacteria will be either dead or so far injured by the NaCl as to be passed restoration.

If spores are present these will be able to resist the action of the NaCl in virtue of their peculiar structure, the addition of Ca will bring about defense rupture action, and it will not matter much if the Ca is injected into the animal at the same time as the spores or much later, as the spores can probably remain alive in the body for long periods. The proper conditions are prepared for their development through the injection of Ca or any other injurious substance that will produce defense rupture.

I have shown in conjunction with Crowe<sup>1</sup>, that the meningococcus can be taken up by leucocytes, but that if these are crushed up seven or eight hours later and the bacteria liberated once more, they are still capable of growth and reproduction. Leucocytes can take up bacteria without being able in all cases to kill and immediately digest them. In the light of this fact, we may conclude that spores with their impervious<sup>2</sup> cell walls survive the attack of the leucocytes and their digestive ferments for a much longer period than vegetative forms of bacteria. Their small size moreover allows of their being readily transported to all parts of the body. We can understand how they may set up an infection at a point where the Ca is injected into the animal ten days after this animal had received the spores themselves.

The difficulty of getting a clear grasp of the relationships of this problem of infection to the gas gangrene organisms in all its aspects, it seems to me is greatly increased in attempting to work it out using spores and vegetative forms of bacteria together. If either are used alone more progress could be made with its solution. In this work of Cramer and Gye, it seems to be forgotten that Ca after all is a violent local poison to the tissue cells of most animals. It is possible to produce abscess formation in many animals with considerable rapidity by the injection of weak solutions of  $\text{CaCl}_2$  in the complete absence of any specific germs. Moreover these authors seem to have surrounded the whole question of the defense rupture action of Ca on the tissues with a certain degree of mystery that is much to be regretted; resolved into its component factors the problem is clear and straightforward.

I have shown that the lethal action of Ca on the cell is irreversible and signifies the commencing death of the cell. The action of NaCl on the other hand is reversible within quite wide limits. The death of the cell in this case takes place very slowly and is due to the diffusion of the salts from the cell; as the cell wall after treatment with a weak but pure solution of NaCl immediately becomes permeable to the free ions in the cell. It is the loss of

<sup>1</sup> Shearer and Crowe (1917), *Proc. Roy. Soc. Ser. B.* LXXXIX. 440.

<sup>2</sup> As illustrating the impermeable character of the spore wall, it is worth recalling Buchner's early observation, where he claimed to have germinated the spores of *B. subtilis* after these had been treated with concentrated sulphuric acid. (See Buchner, "Ueber das Verhalten der Spalt-pilzsporen gegen Anilinfarbstoffe," *Aertzlich. Intelligenzbl.* 1884.)

these salts from the cell, that brings its life to an end<sup>1</sup>. If this diffusion is stopped by transferring the cell to a balanced solution or by the addition of a little Ca to NaCl, then the cell recovers its normal condition, if its loss of salts has not been too great. In distinction to this action that of Ca is final once it has commenced and no recovery is possible<sup>2</sup>.

<sup>1</sup> Since my paper appeared, a clear demonstration of this effect has been described by Gray (*Journ. Physiol.* 1920, LIII. p. 308) in the trout egg. Injury of the cell membrane, whether mechanical or through the poisonous action of salts, immediately renders this membrane permeable to the contained electrolytes of the cell, and the loss of these brings about the precipitation of the globulins of the egg with death; during life these are held in solution by the contained neutral salts.

<sup>2</sup> The recent work of Brooks (*Journ. Gen. Physiol.* 1918-20) on the effect of different electrolytes on the respiration of *B. subtilis* has shown that the action of NaCl and CaCl in affecting respiration is very similar to that demonstrated by the conductivity method as described in my paper.



# REPORT OF EXPERIMENTS ON THE COLD STORAGE OF HERRINGS CARRIED OUT AT NORTH SHIELDS (JUNE AND JULY, 1919)

BY IONE H. GREEN.

(*From Food Investigation Board, Scientific and Industrial Research Department.*)

THE following experiments form part of the general scheme of research undertaken with the object of providing a sound scientific basis for methods of fish preservation and storage.

The bacteriological part of the investigation was first undertaken by the author a year ago and the time has been spent in endeavouring to become acquainted with the peculiarities and characteristics of herring bacteria. There is still a large amount of work to be done in this field before practical experiments on a large scale can be carried out to yield really satisfactory results.

This spring, however, the large cold store at North Shields was put at the disposal of the Food Investigation Board, and it was determined to carry out a few preliminary experiments there.

A store designed for commercial use in the height of the herring season does not lend itself to exact bacteriological investigations. The following report therefore must be considered as dealing with a preliminary survey of a field to be explored more thoroughly later.

## SERIES I. DRY-FREEZING AS COMPARED WITH BRINE-FREEZING.

On June 16th at about 6 a.m. a large number of herrings were trawled 14 miles E. by N. of North Shields. They were of various sizes, but mostly small and apparently underfed, the result being that whether gutted or ungutted their keeping qualities were inferior: all damaged fish were discarded. The herrings under experiment were divided into two lots, one lot being gutted and the other not. At about 11.30 a.m. (8.5 hours after capture) half of the gutted and ungutted fish were put into dry cold-storage, being piled up on trays, at a temperature of 18° F. At the same time the others were put into a brine bath at 10° F. for 1.5 hours. The gutted fish<sup>1</sup> were kept separate from the ungutted by means of a partition dividing the large iron basket in which they were placed. After 1.5 hours they were taken out and put into cold storage with the others. Twenty-four hours later, about ten specimens

<sup>1</sup> Compare with Gardiner and Nuttall (19. i. 1918): Frozen Fish, Dry Freezing, Brine Freezing, *Fish Trades Gazette*, London.

from each lot were removed and placed in trays covered over as protection from the dust and left for another 24 hours at 60°–65° F. They were put where the sun could not shine directly on them. Both dry- and brine-frozen herrings were frozen stiff when taken out of store.

#### OBSERVATIONS.

(a) *Physical examination.* After 24 hours a few of the gutted and ungutted specimens were taken from the dry- and brine-frozen lots and the internal and external appearances very carefully compared. Each day up to the fourth or fifth day they had been exposed to ordinary temperature. The differences were very marked in every way, the brine-frozen being much superior in appearance and keeping qualities. The flesh of the brine-frozen specimens was firm and white after four days and still entirely inoffensive in smell. The flesh of the dry-frozen on the other hand was soft, much greyer in colour and after four days at the same temperature had a very distinct putrefactive smell<sup>1</sup>.

(b) *Bacteriological examination.* Several Petri dishes of fish agar were inoculated with slime from the gills of the dry- and brine-frozen herrings after they had been 24 hours at ordinary temperatures. On examining and comparing these plates after two or three days' incubation, it was found that in nearly every case the plates from the brine-frozen fish showed very much less growth and fewer kinds of colonies than the dry-frozen ones<sup>2</sup>.

This result suggests that either the bacteria are destroyed in larger numbers by the brine method of freezing owing to the lower temperature and the more rapid freezing of the tissues, or that the temperature used in the dry-freezing process was too high to entirely prohibit the slow growth of the bacteria and consequently putrefaction. Unfortunately the cold store was in poor running order at the time and it was impossible to carry out any experiments at a lower temperature than 18° F. whilst the store was often as high as 20°–25° and could never be entirely relied upon.

Taking everything into account, however, it seems there is little doubt that the brine method is the best and most satisfactory, provided the brine tank is kept clean and well filtered. The longer the herrings are kept in it the better, up to four or five hours, so long as the temperature of the brine is never allowed to rise above 10° F.

<sup>1</sup> It should be mentioned that the brine used in the tank for all these experiments was made from fishery salt with the addition of 1·5 % caustic soda and its freezing point was 9° F.

<sup>2</sup> A fuller account of the numerous kinds of bacteria isolated from "dry-stored" and brine-frozen herrings will be given later when more work has been done upon them. At present it will suffice to mention that the bacteria obtained belonged chiefly to the *Proteus*, *B. coli*, *Sarcina*, and Phosphorescent Groups. The *Proteus* Group and Phosphorescent forms were the most prevalent on the whole.



SERIES II. BACTERIOLOGICAL EXAMINATION AND COMPARISON OF  
HERRINGS BRINE-FROZEN, DRY-FROZEN, AND FRESH.

Samples of brine- and dry-frozen fish were brought out of store and placed on trays with an equal number of perfectly fresh herrings straight from the drifter. These were left away from the sun and wind at a temperature of about 60° F. for two or three days and then bacteriological samples were taken from them and inoculated into fish broth.

OBSERVATIONS.

- (a) Brine-frozen herrings produced the least amount of bacterial growth.
- (b) Dry-frozen herrings produced by far the greatest amount of growth.

This experiment, like the first, shows that bacteria do continue to multiply at a temperature of 18°–20° F., whereas the brine-freezing method not only inhibits growth but kills many of the forms of bacteria present before they have time to spore.

SERIES III. APPEARANCE AND KEEPING QUALITIES OF HERRINGS GUTTED  
BEFORE AND AFTER BRINE-FREEZING AND LEFT UNGUTTED.

Having shown that under the above conditions brine-freezing is the most satisfactory, the following experiments were carried out using this method only. Several gutting experiments were made during the next three weeks under the following headings:

- A. Comparison (physical and bacteriological) of frozen herrings gutted before freezing and left ungutted.
- B. Comparison (physical and bacteriological) of frozen herrings gutted before freezing and gutted after and exposed for a few days to ordinary temperature.
- C. Comparison of herrings gutted in the normal commercial way and gutted by means of searing irons.
- D. Bacteriological examination and comparison of herrings gutted and ungutted while still in a frozen state.

A.

Comparison of frozen herrings gutted and ungutted  
before freezing.

A large number of fish straight off the drifter were placed, gutted and ungutted, in brine and dry-frozen for a week at an uneven temperature between 18° and 25° F. The largest and best looking specimens were then chosen out of this lot, each placed on trays, and left lightly covered from the dust for four days at a temperature of about 60° F.

## OBSERVATIONS.

(a) *Physical examination.* Altogether about 50 gutted and ungutted fish were examined carefully externally and internally, and comparisons made. Each fish was cut down the length of the spine and opened out like a kipper for inspection. Little difference in external appearance could be detected between gutted and ungutted fish but much difference was apparent internally. Comparisons were best made after the fourth day at ordinary temperature.

The flesh of the ungutted herrings was still fairly white, firm, and in most cases free from any putrid smell. The muscle tissue immediately round the spine was inclined, on the whole, to be more bloodstained than was the case with the gutted ones, but in spite of this the flesh was firmer, whiter and in better condition.

The flesh of the gutted ones, on the other hand, was much yellower and more discoloured although not so bloodstained down the spine. They also had a very strong oily smell which was completely lacking in the ungutted ones, in fact the general appearance and smell of them was not nearly so good.

(b) *Bacteriological examination.* A large number of fish-agar Petri dishes were inoculated with samples of muscle from gutted and ungutted herrings after four or five days at ordinary temperature and comparisons were made of the amount of bacterial growth produced. The result was that in about two-thirds of the total number of plates the largest amount of growth came from gutted herrings. In view of the fact that the bacteriological samples were taken with extreme care in every case and the muscle always taken from the same part of the fish, it was proved pretty definitely that more bacteria made their way into the muscle tissues of the gutted than of the ungutted specimens.

This result is due to the gutting process wherein a large amount of gut contents are left behind upon the inner body wall, and, although much of it is washed off in the brine tank, a large number of gut bacteria penetrate the flesh and start putrefaction more quickly than in the case of the ungutted fish, where the bacteria are imprisoned within the gut whose wall has first to be penetrated. Moreover in the case of the gutted fish the inner as well as the outer surface is exposed to foreign contamination of all sorts, particularly after coming out of store, and this certainly hastens putrefaction more than when only the outer surface of the fish is open to infection.

It ought perhaps to be mentioned that the colonies produced on all the plates were chiefly of the same kind, namely moist, round, buff-coloured, fairly large, irregular sized, Gram-negative diplococci. All the plates gave off a very strong smell of ammonia after the third day.



## B.

## Comparison of frozen herrings gutted before and after freezing.

Several gutted and ungutted brine-frozen herrings were brought out of storage. The ungutted ones were gutted as soon as they were thawed and placed on trays alongside the already gutted ones and left in a clean place at a normal temperature of about 60° F. for a few days. They were examined carefully each day during this period and at the end of four days fish-agar Petri dishes were inoculated with samples of muscle from each kind of herring including ungutted ones as controls.

## OBSERVATIONS.

No very definite conclusions could be formed from this experiment. The appearance and keeping qualities of the fish gutted before and after freezing seemed the same. But there is no doubt that gutting after freezing is neither so practical nor so clean, for the gut during the process of freezing becomes brittle and easily broken. It is therefore difficult to remove it entirely and any gut left behind does more harm than if the whole had been left undisturbed.

The amount of bacterial growth produced on the plates was about equal.

## C.

## Comparison of herrings gutted in the usual way and by means of searing irons.

A dozen or so of the finest and largest herrings were obtained straight off the drifter, they had been dead about seven hours and were in perfect condition. Half of them were immediately gutted in the usual way and the other half gutted by means of hot searing irons, taking great care that in each case after gutting the inner body wall was seared all over to exterminate any gut bacteria which might have been left behind. The two lots were put into the brine tank and frozen for 1·5 hours, then taken out and left in cold storage for 24 hours. The next day they were brought out and placed as usual on trays protected from the dust and left at ordinary temperature for three or four days. At the end of this time they were carefully examined, and physical and bacteriological comparisons made.

## OBSERVATIONS.

(a) *Physical appearance.* Very little difference was to be detected in the internal and external appearance and keeping properties of the two kinds. On the whole the seared specimens were not in quite such good condition but perhaps this was due to their having been handled more.

(b) *Bacteriological examination.* Plates of fish agar were inoculated with pieces of muscle by means of carefully sterilised instruments and comparisons

made of the amount of bacterial growth produced. It was found that the plates inoculated with the seared fish gave the least amount of growth.

This experiment also helps to sustain the contention that the ordinary method of gutting does more harm than good on account of the gut bacteria left behind.

#### D.

### Bacteriological examination and comparison of herrings gutted and ungutted while still in a frozen condition after brine-freezing and cold-storage.

Several fish-agar plates were inoculated with muscle samples from gutted and ungutted herrings immediately they were removed from the cold store and before they were allowed to thaw. The samples were taken from three different lots of fish and put into store for different periods, viz.:

- (1) Brine-frozen 1·5 hours, 25 days in cold store;
- (2) Brine-frozen 2 hours, 18 days in cold store;
- (3) Brine-frozen 4 hours, 11 days in cold store.

#### OBSERVATIONS.

A large amount of bacterial growth was produced on all the plates inoculated from gutted specimens and much less growth on the plates from ungutted fish. This again leads to the conclusion that putrefaction is slowly going on while the fish are in cold store or at any rate that the bacteria are slowly multiplying. Regarding the effect of the cold store on the growth of the bacteria, the result obtained was doubtful, as one set of experiments showed the longest stored fish to have produced the greater amount of bacterial growth, the other set of experiments showed the reverse. It is possible, however, that the latter result was due to contamination of the plates and unfortunately there was no time to repeat the work.

It had been intended to carry out experiments on non-oily fish for comparison with those on herring above described, but unfortunately during the time available at North Shields only the herring drifters were going to sea, the trawlers being on strike. Fish could have been obtained from other ports but it would not have been fresh enough for the purpose.

It would have been of extreme interest and importance to note if non-oily fish of the same size as herrings, when treated in the same way, gave similar results. It is well known that herrings disintegrate and deteriorate more rapidly than other fish, but whether herrings are the more rapidly attacked by bacterial growth or not remains, in my opinion, to be elucidated. The flesh of the herring is very delicate and therefore more quickly and easily attacked and altered in autolysis, and moreover the oil of the herring very quickly discolours the flesh and becomes rancid. This was particularly noticeable in herrings which had been gutted, thus giving the fish the



appearance of having started to putrefy before this was strictly the case bacteriologically.

#### CONCLUSIONS.

The general conclusions from the investigations at North Shields are:

(1) That brine-freezing is more satisfactory than dry-freezing at 18° F. both for gutted and for ungutted fish.

(2) That the keeping properties of ungutted frozen fish are superior to those of the gutted ones.

(3) That if herrings are to be gutted it is cleaner and more practical to gut them before freezing.

It is very regrettable that some of the experiments carried out lose much of their value because air temperatures lower than 18° F. were not available. It is very unlikely that any temperature above 14° F. will entirely prohibit the growth of putrefactive fish bacteria. Hence a store run at 18°–20° F. will only serve to retard the process of putrefaction, and, owing to the slow multiplication of bacteria in fish thus stored, the keeping properties of the fish after removal from cold storage will be much inferior to that of freshly caught fish.

It is interesting to note that the gutting of warm-blooded animals before cold-storage has also been found to be unsatisfactory. Miss Pennington gives an account of this in her paper on "The Handling of Dressed Poultry a Thousand Miles from the Market<sup>1</sup>." She states that poultry should not be eviscerated until they have arrived at their destination and are about to be cooked, although she does not give the scientific reason for this recommendation. It was doubtless found, as in our case, that the eviscerating process, although it can be effected in a cleaner and more scientific way than is possible with small fish, gave rise to more rapid bacterial growth and relatively quicker putrefaction. In dealing with poultry it might well be anticipated that the difference in the results obtained by the two methods would be much more marked than was the case with herrings, as the advantage of being able to clear out the intestinal tract by special treatment just before the birds were killed is a very important factor. It is well known of preserved fish that the keeping properties of full gutted specimens are not nearly so good as in specimens whose gut is nearly empty, provided the fish is in otherwise good condition. If some practical method could be found by means of which the fish, instead of being gutted before cold storage, could be thoroughly cleared out, that is, if the intestinal contents could be removed without internal or external injury it might be confidently anticipated that the keeping properties would be still further improved.

Dr Prince of the Canadian Biological Board gives a good account of how sea fish should be cold stored and handled on a large commercial scale, and recommends that all large fish should be gutted before freezing, but that

<sup>1</sup> *Yearbook for 1912*: U.S. Department of Agriculture.

small fish can remain ungutted. It would be interesting to know if he has found by scientific investigation that small fish *only* keep better when cold-stored in an ungutted state. Speaking from a bacteriological point of view and without special experience, the author would anticipate that the same rule would apply to large fish such as cod, salmon, etc., as in the case of small fish like herrings.

#### GENERAL OBSERVATIONS.

Before the cold storage of herrings or of any other kind of fish, whether stored in an ungutted state or not, can become a really satisfactory proposition, it is absolutely essential that more cleanly methods should be employed. The present methods of handling and packing herrings in our country are open to the strongest criticism from the bacteriological point of view. As the author has personally observed, the fish is handled as it arrives on the quay without any attempt at cleanliness. The fish, if "washed" and gutted before being exported, are dipped quickly into a slimy liquid culture of mixed fish bacteria (so-called clean water), and, in the case of herrings, they are packed with ice or ice and salt in large deep barrels previously soiled with a layer of slime and scales from many thousands of other herrings which have been packed in the same barrels over a period of months or even years! The herrings become thoroughly contaminated in this way and, however fresh, are rendered liable to quick putrefaction; they are afterwards conveyed to various parts of the country, or, if there is a glut, are sent to the local or nearest cold-storage plant which is probably being run at a fluctuating temperature of anything between 20° and 30° F. The final result of all this is, that if the weather is hot, tons of fish arrive at their destination either unfit for food or in a very doubtful state.

The cost of organising proper machinery for preventing this waste of valuable food, would in a short time be repaid tenfold by the enormous saving effected. If at present no practical method can be devised for equipping drifters with their own brine-freezing apparatus, there is at any rate no reason why every fishing port should not have its own cold storage on the quay ready to receive fish directly it is landed and run at a constant temperature of 14° or at most 15° F. If all fish to be exported to other towns were frozen directly after landing by being put into brine tanks in cold store, and then packed for export in well insulated boxes whilst still in a frozen condition, we should not only save the fish landed during a glut but it would arrive at its destination in a condition so much superior to that of the unfrozen fish that the public would not only lose their prejudice against frozen fish but would soon come to prefer it.

Another point is that herrings whether previously frozen or not should never be packed in deep barrels but in shallow cases. When packed carelessly in deep masses they deteriorate much more easily. They get bruised and therefore are more liable to putrefaction. Also the boxes in which they are



packed ought never to be used a second time without being thoroughly swilled out, or better still, scrubbed out with soap and water to which some antiseptic has been added. The bloody slime and scales which stick to the sides of the boxes act as a splendid breeding ground for the bacteria, and should never be allowed to accumulate.

Finally, if fish when gutted cannot be washed in *running* water, they are far best left unwashed altogether. When public opinion demands that these elementary measures of cleanliness should be carried out and effective means of supervision are provided, then, and not until then, will the Fishing Industry benefit fully by the solving of such scientific problems as arise in connection with the cold storage methods of preservation.

I desire to express my best thanks to Mr Wilson at the cold store at North Shields and to the authorities at the School of Medicine, Newcastle, for the kind assistance given during the work. I have also had an able assistant in Mr G. Ll. Rogers.

## AN ENQUIRY CONCERNING THE STATE OF CLEANLINESS OF EMPTY MILK CHURNS.

BY R. H. CUMMING, B.Sc., AND A. T. R. MATTICK, B.Sc.

*(From the Research Institute in Dairying, University College, Reading.)*

AN enquiry (1) instituted in 1916 showed that from 1 to 2 % of rail-borne milk was sour on arrival at its destination, with the result that in London alone there was a loss of some 90,000 gallons of milk annually. That this loss was not confined to the London milk was brought out by the fact that a similar loss was found to obtain in the north of England.

Further investigations (2) showed that average milk, when carried in utensils and churns washed according to the methods generally obtaining, had a very high bacterial content especially during the summer months. The examinations for bacterial content were made when the milk was not more than about nine hours old. Proliferation of the organisms introduced into the milk at the time of milking was not therefore a sufficient explanation of this condition. It was thought that possibly an examination of the churns which were being returned empty to farmers might reveal one source of this excessive number of bacteria, since if the churns were inefficiently cleansed and particularly if they were also left damp, every opportunity would be afforded for the proliferation of bacteria during the warm summer weather, and the churns would reach the farmer in such a state that it would be very difficult for him to cleanse them adequately, even supposing he were in possession of the necessary apparatus.

For these reasons therefore the following series of experiments was undertaken.

Between October 9th and November 20th, 1919, 500 empty milk churns were examined for cleanliness on a station platform.

The state of cleanliness was measured by the following methods:

- (A) The general cleanliness as revealed by inspection.
- (B) The state according to the smell.
- (C) The number of colonies found by a bacteriological examination of the washings with sterile normal saline solution.

The results of (A) and (B) are set out in Table I in which the churns are classified according to their state as determined by inspection and the presence or absence of smell.

Table I shows that only 16 % of the churns examined were apparently clean and dry and 28 % though, apparently clean, were wet. On the other hand, 56 % of the churns were undoubtedly in an unsatisfactory condition, since all of them revealed one or more of the following faults: the presence



either of milk or of milky water, or of an evil smell. In addition to the examination by inspection, bacteriological examinations of churns of each type were carried out by the following methods. One litre of sterile normal saline solution was poured into each churn which was examined and was thoroughly agitated,—a portion being first poured on to the inside of the lid

Table I.

Classification of churns by inspection.  
October 9th—November 20th, 1919.

Clean and sweet		Milk absent. Evil smell		Churn not washed. Milk present	Churn badly washed
Dry	Wet	Dry	Wet		
81	140	5	118	81	75
16%	28%	1%	24%	16%	15%
Total = 500					

and then added to the bulk. A sample of the washings was then taken into a sterile bottle and examined bacteriologically within one hour of collection, by plating dilutions up to one in a hundred thousand on neutral lemco agar, incubating the plates for five days at 22° C. and then counting the number of colonies which grew. Litmus lactose peptone water tubes were also inoculated with 1 c.c. of the washings, and 1 c.c. each of the dilutions, and incubated for five days at 37° C. These were then examined for the presence or absence of lactose fermenting organisms as shown by the production or non-production of acid and gas.

The results of these examinations are shown in Table II, p. 86.

On analysis, this table shows that those churns which contained milk or milky water showed very high bacterial counts, and lactose fermenting organisms were found in high dilutions in all cases. Churns of the clean and dry type showed counts both high and low, but it is noticeable that the lactose fermenting organisms when present were not generally found in such high dilutions as in the case of samples from churns containing milk or milky water. Several of the churns of this type which were examined, showed counts which were well under 10,000 per cubic centimetre of washings and lactose fermenting organisms were not found. On the other hand very high counts were obtained in some cases in this series showing that, although these churns appeared to be clean and were dry, they had not really received an efficient cleansing.

Out of a total of 13 churns of the clean and dry type which were examined bacteriologically, only 5 showed really low counts and contained no lactose fermenting organisms, so that although these churns appeared to be clean and would readily have passed a cursory examination, there must have been some grave defect in the methods of cleansing, which either did not eradicate or introduced contaminating organisms in the majority of cases.

Some idea of the gross contamination of milk by dirty churns may be gathered from a consideration of the figures in Table II.

Table II.  
Bacteriological Examination.  
October 9th—November 20th, 1919.

Condition of churn	Counts	Acid and gas in litmus lactose peptone water
Unwashed (whole milk present)	Uncountable in	
	1/100,000	+ 1/100,000
” ” ”	4,000,000	+ 1/100,000
” ” ”	Uncountable in	
	1/10,000	+ 1/10,000
Badly washed (milky water present)	18,400,000	+ 1/1000
” ” ” ”	5,300,000	+ 1/1000
Apparently clean but wet	4,100,000	+ 1/100
” ” ”	780,000	+ 1/10,000
” ” ”	510,000	+ 1 c.c.
Apparently clean and dry	6,200 000	+ 1/1000
” ” ”	1,720,000	+ 1/10,000
” ” ”	1,600,000	+ 1/100
” ” ”	800,000	+ 1 c.c.
” ” ”	400,000	+ 1/1000
” ” ”	35,000	+ 1/10
” ” ”	15,400	+ 1 c.c.
” ” ”	6,500	—
” ” ”	5,000	+ 1/10
” ” ”	3,000	—
” ” ”	2,400	—
” ” ”	2,000	
” ” ”	170	—

The best of the churns contained 170 bacteria per cubic centimetre of washings. Therefore as one litre of saline solution was used for washing, there must have been at least 170,000 bacteria in the churn. Another churn was found to contain at least 18,400,000 bacteria per cubic centimetre of washings. If 17 gallons of milk were put into this churn, that milk would start on its journey to the factory or consumer with an initial contamination of approximately 240,000 bacteria per cubic centimetre. These are the results of an examination in winter, when temperature conditions were not favourable for the rapid proliferation of organisms. It is probable that in summer, milk such as this would arrive at the end of a long railway journey in such a condition as to be unfit for use.

The investigation proves that the present methods of washing and cleansing churns are inadequate and one cause of the loss of milk through souring in transit. Further experiments are being undertaken to establish the minimum time of steaming necessary to insure as complete a sterilization as is possible under practical conditions.

REFERENCES.

(1) WILLIAMS, R. STENHOUSE (1917). Wastage of Milk. *Journ Roy. Agric. Soc.* LXXVIII.  
(2) FREEAR, K., BUCKLEY, W. and WILLIAMS, R. STENHOUSE (1919). *Memorandum concerning Two Types of Commercial Milk*. Research Institute in Dairying, University College, Reading.



SUGGESTIONS FOR A NEW CRITERION OF A  
POSITIVE WASSERMANN REACTION BASED ON  
AN ANALYSIS OF 2334 QUANTITATIVE TESTS<sup>1</sup>.

By C. H. BROWNING AND E. L. KENNAWAY.

(From the Bland-Sutton Institute, Middlesex Hospital and the Pathological  
Department of the University and Western Infirmary, Glasgow.)

(With 3 Text-figures.)

CONTENTS.

	PAGE
I. Introduction . . . . .	87
II. Method of Analysis of Records . . . . .	89
III. The Negative Control Serum . . . . .	93
IV. Technique employed in the Tests . . . . .	94
V. Method of Recording Degrees of Lysis . . . . .	95
VI. Discussion of Results. A new Criterion of a Positive Reaction . . . . .	96
VII. Confirmatory Evidence:	
(a) Series III . . . . .	97
(b) Comparison of the proposed method of diagnosis with that previously used . . . . .	101
VIII. The Application of the Results in Diagnosis . . . . .	102
Summary . . . . .	104
Note I. The Amounts of Complement used in Wassermann Tests . . . . .	105
Note II. Proportions of Positive and Negative Sera . . . . .	105

I. INTRODUCTION.

THE criterion of a positive result in the Wassermann test, which is commonly adopted, is that a given amount of complement, which along with a known negative serum and the “antigen,” causes complete lysis of the test corpuscles, will with a positive serum in the same combination show no lysis or, at most, a faint trace, i.e. lysis not exceeding about 30 per cent. of the corpuscles. This may be termed the “all or nothing” criterion. Some workers adopt a fixed volume of guinea-pig’s serum, e.g. in the original method of Wassermann and his co-workers, but it is to be remembered that the amount which these observers employed at first was subsequently reduced by half. Others (e.g. Boas, Fildes and McIntosh) employ a definite number of haemolytic doses of complement. The results thus obtained, although they afford useful indications, are by no means perfectly satisfactory. Thus Wassermann’s one tube method neglects the following facts: (1) that the haemolytic power of different

<sup>1</sup> A Report to the Medical Research Committee.

guinea-pigs' sera may vary considerably; (2) that there is no fixed relationship between deviability and dosage (see Table III); and, further (3) that since only one amount of complement is used, it is not possible to differentiate degrees of positiveness satisfactorily. With regard to the last-mentioned point, the range of lysis obtained with the mixture of patient's serum and antigen along with varying amounts of complement is not necessarily similar with different patients' sera (see Table I); also, owing to the comparatively large amount of complement employed by Wassermann, it is probable that a number of the less pronounced, but still significant, positive reactions are missed. This is a very serious defect when employing the reaction in the control of treatment or when cases are examined at an early or latent stage of the infection. Boas, in order to introduce a quantitative gradation, employs a series of varying amounts of patient's serum along with a definite number of haemolytic doses of complement, and this procedure was also adopted by Fildes and McIntosh; here again variations in the deviability of different specimens of complement are neglected. Latterly Fildes and McIntosh have used only a single amount of serum. All of the criteria employed above, therefore, are unsatisfactory in as much as they fail to allow for the varying sensitiveness of complement to the reagents, antigen and patient's serum, separately or together. Accordingly, it is necessary to discard the results when the control tests show that the complement is abnormal in its behaviour.

Browning and Mackenzie (1909) showed at a comparatively early period that when a given serum was tested with two different specimens of complement, all the other reagents being identical, the degree of positiveness as ascertained by the two series might show a marked difference. Recently Browning and Kennaway (1919) have reported the results obtained when the same specimen of syphilitic serum was examined for complement fixation in the Wassermann test on a number of different occasions and have found that: (1) the actual amounts of complement fixed, reckoned either by volumes or as haemolytic doses, varied greatly on the different occasions, and (2) the variations were quite irregular, and depended on factors which cannot, so far, be rendered constant. Thus it was not possible to fix upon any single amount of complement the behaviour of which, along with "antigen" and patient's serum, would determine whether that serum was positive or negative. In the modified original technique of Wassermann 0.05 c.cm. of complement was employed, under specified conditions, in all cases; sera showing no lysis with this standard amount were regarded as positive, and those showing complete lysis as negative. But the results with the positive serum obtained by Browning and Kennaway showed that on one day 0.04 c.cm. complement sufficed to produce commencing lysis, whereas on other occasions no less than three times this amount was required. Browning and Mackenzie (1911) adopted originally as the standard of a positive serum that along with the antigen it should require at least five doses of complement in addition to the sum of the amounts inhibited by the serum and the antigen separately,



in order to produce complete lysis of the test corpuscles. These workers also specified the limits of inhibitory effect on complement which each constituent might exercise by itself without disturbing the results of the test. A fixed amount of patient's serum and a series of varying doses of complement were accordingly employed. The method, however, appears to have been regarded as too complicated to commend itself generally and the number of tubes originally recommended (five or six) was such as to consume too much time in the performance of the test. It is obvious, however, that for a satisfactory method of performing the test it is necessary to institute a quantitative comparison between the complement-inhibiting property of the mixture of patient's serum and antigen on the one hand and a known negative serum along with antigen on the other. Accordingly, the object of the present investigation was to ascertain whether the study of the numerical results of a large number of Wassermann tests in which the actual amount of complement fixation was determined, would suggest any rule, more satisfactory than those already in use, by which the distinction between "negative" and "positive" sera could be defined. Clearly no system can wholly eliminate those reactions which must be reported as "suspicious," "doubtful," or " $\pm$ ," since the serum of every patient who has recently become infected, or who is passing into the state of latent infection either as the result of treatment or spontaneously, must at some period be in transition from the negative to the positive condition, or *vice versa*. But for purposes of clinical diagnosis it is well to determine how far the use of such terms may be restricted to a minimum.

## II. METHOD OF ANALYSIS OF RECORDS.

With the above object in view, the quantitative records of over 2000 Wassermann reactions were expressed in graphic form, in the hope that the curve of distribution thus obtained would give some indication of the point at which the transition from negative to positive occurs. Three series of records were examined, comprising 2334 sera in all; Series I contained 540 sera, Series II, 533, and Series III, 1261. A fourth series of 508 sera is also utilised in the last section of this paper. The results in the first series were obtained during 55 days of routine testing, those in the second series during 37 days, and those in the third during 69 days. The first, third and fourth series comprise specimens of blood submitted for diagnosis from the wards and out-patient departments of a general hospital; a certain number of cases which have undergone anti-syphilitic treatment are, of course, present among these. We are indebted for Series II to the records of Dr H. Ferguson Watson; the cases were examined by him and were quite independent of the other series. Further, in Dr Watson's series there are certainly very few, if any, treated cases, as the specimens were taken from children and adults with a view to determining the prevalence of syphilis and not in order to diagnose the nature of diseased conditions.

The records in Series I and II were dealt with in the following manner. The amount of complement required to produce just complete lysis with the negative control serum in the presence of antigen is reckoned as 100. The amounts of complement required to produce just complete lysis with each of the other sera tested upon the same occasion can then be expressed as percentages of the amount required by the negative control serum. In this way a quantitative comparison of the character of the different sera is obtained. Further, if the point of initial lysis, as well as that of complete lysis, can be observed, the amount of complement required to complete the haemolytic process when once begun can be expressed upon the same system of percentages. This latter amount is remarkably variable even in groups of sera which require the same amount of complement to produce complete lysis; some will show a short, others a long range of partial lysis. An example is given in Table I below; in this, the range with one serum (*N*) is about twice as great as it is with the other (*A*). This feature is obviously a subject for study, although its significance is by no means clear.

Table I.

*Example of difference in range of lysis with different positive sera tested with the same complement. Antigen, liver-lecithin + cholesterol.*

Date: 15. x. 12.

Complement c.c.	0.024	0.035	0.05	0.07	Serum control	
					0.01	0.02
Serum ( <i>A</i> )	None	None	Trace	Complete	Almost complete	Complete
Serum ( <i>N</i> )	Faint trace	Trace	Almost complete	Just complete	Almost complete	Complete

One point of great practical importance immediately arises out of this fact, however, viz. the unsatisfactory character of any method which employs a single amount of complement, e.g. Wassermann's original procedure. In examining a series of quantitative tests it is easy to pick out sera which along with antigen give marked or complete lysis in a mixture containing 0.05 c.c. complement and which, therefore, would be returned as negative; but a quantitative examination shows that they give little or no lysis with 0.03 c.c. complement, whereas the negative control is complete with 0.01 c.c.

The application of the method of calculation described above may be illustrated by the following examples (Table II). The amount of complement required to produce just complete lysis with the negative control serum (0.015 c.c.) is taken as 100; the amounts in the other three tubes (0.03, 0.045, and 0.06 c.c.) are therefore represented by 200, 300, and 400. With serum *A*, lysis begins at some point below 100, and extends to 200. With serum *B*, lysis begins near 200 and extends to 400. With serum *C* lysis begins near 300 and extends beyond 400, the whole range not being observed. These results may be represented graphically as in Fig. 1. The arrows indicate that the complete range of lysis could not be observed within the series of tubes employed.



Table II.

Tube	(1)	(2)	(3)	(4)
Amounts of complement in c.c.	0.015	0.03	0.045	0.06
Doses . . . . .	2	4	6	8
Percentage . . . . .	100	200	300	400
Negative control serum . . .	<sup>1</sup> Just complete lysis	Complete lysis	—	—
Serum A . . . . .	Very marked lysis	<sup>1</sup> Just complete lysis	Complete lysis	
Serum B . . . . .	No lysis	Trace of lysis	Distinct lysis	<sup>1</sup> Just complete lysis
Serum C . . . . .	No lysis	No lysis	Trace of lysis	Distinct lysis

<sup>1</sup> I.e. the fluid is nearly but not perfectly clear, and a trace of red corpuscles, amounting to about two per cent. of the total quantity originally added, is visible at the bottom of the tube on standing.

Haemolytic dose of guinea-pig's complement for 0.5 c.c. 3 per cent. suspension of ox or sheep corpuscles sensitised with at least 5 doses of immune body = 0.0075 c.c.

The great advantage of this method of representing the results is the fact that the behaviour of the known negative serum constitutes its basis<sup>1</sup>. This must always be the chief guide in diagnosis, since it serves as a control upon the variations which occur both in the deviability of complement (in the direction especially of over-deviability)<sup>2</sup> and in the complement-absorbing power of different samples of antigen. However, when any large series of

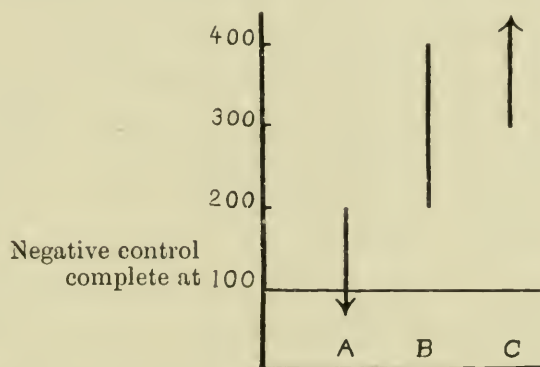


Fig. 1.

results is dealt with in this way the following difficulties, omitted purposely from the examples given above, are encountered. (1) The negative control may show quite complete lysis in the first tube; the base-line (100) cannot then be exactly ascertained, as the amount of complement required to produce just complete lysis is unknown. The results obtained on such days are, therefore, not perfectly suitable for presentation by this method. (2) In a large proportion of cases, the points of initial and of just complete lysis cannot be observed; they will either lie outside the series of tubes, as in sera A and C

<sup>1</sup> Considerable importance attaches to the choice of a negative control serum. This point is discussed in detail on p. 93.

<sup>2</sup> The use of a known weak positive control serum is chiefly of value for controlling the deviability of the complement in the direction of under-deviability (see p. 104).

above, or they will, so to speak, fall between two tubes. A number of tubes impracticable in routine work would be required in order to obtain results ideally suitable for statistical purposes. Suppose for instance, that lysis is well advanced, but by no means complete, in one tube of the series, and is quite complete in the next; where is the point of just complete lysis? The point in question can be expressed as a percentage only by a process of guessing which cannot be dignified by the term "interpolation." If all sera exhibited the same range of lysis (i.e. required the same amount of complement in order to cause the same increment of lysis, e.g. from distinct to very marked), and differed only in the region of the whole scale in which this range lay, it would be possible to interpolate in a satisfactory manner; but, as was pointed out above (see p. 90), this extension of partial lysis is a most variable character. In practice one must judge as best one can, from the rate of advance of lysis seen in the preceding tubes, whereabouts the point of just complete lysis would lie. An alternative method which would eliminate guessing, would be to take as "just complete" always the first tube showing "quite complete" lysis; but owing to the necessarily large intervals (generally 100 per cent.) between the amounts of complement employed in successive tubes, this would seem to involve a greater error than does guessing, at any rate in cases where lysis is far advanced in the preceding tube. (3) As one cannot in practice measure and record all the different degrees of partial lysis which occur, the results fall inevitably into groups which do not give a strictly accurate representation of the facts. These degrees of partial lysis are, of course, infinite in number, and could be estimated with accuracy only by the examination of the contents of each tube in a haemoglobinometer, which is not practicable. Accordingly, in recording the results of Wassermann reactions we employ only eight terms to denote the stages of lysis; the quantitative significance of these terms is discussed below (p. 95). The grouping which results from this necessary limitation in the number of descriptive terms is evident in the general form of the curves shown in Figs. 2 and 3. However, this source of error tends to produce a series of flat segments or steps in the distribution curve, and one must, therefore, attach the more significance to any portions of it which show a gradient. Thus, it is evident that the records of Wassermann reactions cannot yield material which is thoroughly suitable for statistical purposes unless (1) a very long series of tubes be used, and (2) the degrees of lysis be estimated by some accurate method. Since it is impracticable to realise these conditions in routine work, one must either abandon examination of the abundant material which is at hand or make what use of it one can in its relatively defective state. In view of the importance of the question involved and of the fact that we are probably in possession of the fullest data available on the subject, we have judged it advisable to make a comprehensive examination.



## III. THE NEGATIVE CONTROL SERUM.

The quantitative method of performing the Wassermann reaction proved clearly at an early stage that all negative sera are not alike in their behaviour. Thus in Figs. 2 and 3, a number of sera are represented at the left-hand side of the graph as lying below the horizontal line at 100; that is to say, they are more negative than the negative control and the majority of the negative sera. They amount to 54 out of the 760 sera in the two figures, and constitute 7 per cent. of the whole number, or 11 per cent. of the negatives (i.e. those showing complete lysis at or below 150). On the other hand, some non-syphilitic sera, including those from certain adults who are apparently in good health, will tend to fix more complement in the presence of antigen than the great majority of other negative sera do. To these sera we have applied the term of *border-line negatives*. They are not by themselves specially anti-complementary. But with a hyper-sensitive complement such sera will yield an apparently positive reaction, while in the same series many other sera will react definitely negative; this is illustrated in Table III. The experiments

Table III.

*Behaviour of different non-syphilitic sera tested simultaneously with two complements, illustrating an extreme degree of over-deviability in Complement II. Antigen, liver-lecithin + cholesterol.*

Complement, c.c.	0.015	0.025	0.04	0.06	0.09	Serum control 0.015
<i>Complement I</i> (M.H.D. .005 c.c.)						
Negative control serum <i>A</i>	—	Very marked	Complete	—	—	Complete
Negative control serum <i>B</i>	—	Complete	—	—	—	Complete
Emulsion control	Complete					
<i>Complement II</i> (M.H.D. .005 c.c.)						
Negative control serum <i>A</i>	—	None	None	Very marked	Complete	Just complete
Negative control serum <i>B</i>	—	Faint trace	Trace	Very marked	Complete	Complete
Patient's serum <i>C</i>	—	Complete	—	—	—	Complete
Emulsion control	Complete					

recorded in this table were carried out upon the same day with two samples of complement (I and II) each obtained from the pooled blood of two guinea-pigs and treated in the same manner before use; all the other reagents employed were the same throughout the series. While both complements showed a normal behaviour in the serum and antigen controls, and had the same M.H.D. (0.005 c.c.), Complement II showed extreme over-deviability in the presence of sera *A* and *B* together with antigen, while another serum (*C*) gave a typical negative result. The negative character of sera *A* and *B* is known from many years' experience, yet with Complement II they require no less than eighteen M.H.D. to give complete lysis. Complement II represents

an excessive degree of over-deviability, which in our experience is extremely rare; but the results illustrate what is, in a less pronounced form, very common.

We have, therefore, attached great importance to the use of such a negative serum and have for several years past constantly employed as the negative control a serum of this kind derived from one individual. Its normal behaviour thus becomes known, and any unsuitability in the other reagents is the more readily detected, and if not too great, allowed for. A stock sufficient for several months can be kept frozen in amounts each suitable for a single day's work, whereby repeated thawing is avoided<sup>1</sup>. In this laboratory we have now used the serum of one person upon 355 occasions. This serum constitutes the negative control in Series III (heart-cholesterol antigen) and IV and on six of the 55 occasions in Series I; in Series II for which the serum in question was not available, the negative control differed from day to day and was not selected on the above principle. The results in Series II indicate that the conclusions arrived at in this paper regarding the criterion of a positive reaction hold generally and are not dependent on the use of any particular negative control serum. Thus when the use of a single serum, such as that described above, is impracticable we would suggest that *the negative control should consist of a pooled specimen of at least twelve negative sera derived from cases which have not recently received anti-syphilitic treatment and in which there is no question of early syphilis before a positive reaction has developed.*

#### IV. TECHNIQUE EMPLOYED IN THE TESTS.

All the Wassermann tests of which the records were utilised in Series I and II were carried out by the method of Browning, Cruickshank and Mackenzie. Two antigens were employed, namely a solution of ox-liver "lecithin" and the same with the addition of 1·2 per cent. of cholesterol; one volume of the alcoholic solution was mixed slowly with seven volumes of saline, and each tube received 0·3 c.c. of this emulsion together with 0·025 c.c. of patient's serum. Only those results obtained by the use of the latter antigen are dealt with here, as the process of drawing any conclusion is very much complicated if two figures have to be considered in the case of each serum. In Series III, the antigen was a rapidly mixed 1 in 30 dilution of a mixture of 3 volumes of alcoholic extract of human heart and 2 volumes of a 1 per cent. solution of cholesterol, i.e. it corresponds to the antigen employed in the method of Fildes and McIntosh. Each tube received 0·5 c.c. of emulsion and 0·05 c.c. of patient's serum. The haemolytic system consisted of 0·5 c.c. 3 per cent. suspension of washed ox (or occasionally sheep) blood sensitised with at least 5 doses of immune body from the rabbit<sup>2</sup>.

<sup>1</sup> The temperature was maintained at  $-15^{\circ}\text{C}$ . or lower. It is possible that temperatures more closely approaching  $0^{\circ}\text{C}$ . may be less suitable owing to the occurrence of autolytic changes. This point requires further investigation.

<sup>2</sup> For further details of the methods see Browning and Watson (1919), *Venereal Diseases*. London.



## V. METHOD OF RECORDING DEGREES OF LYSIS.

The various degrees of lysis which occur in the series of tubes employed in the test have been described in the records by an arbitrary series of terms, namely: *faint trace*, *trace*, *distinct*, *marked*, *very marked*, *almost complete*, *just complete*, *complete*. In order to ascertain the range of lysis over which the observers concerned would apply each of these descriptive terms, a number of experiments with mixtures of known proportions of lysed and unlysed corpuscles were made in the following manner: 3 c.c. of washed red corpuscles are mixed with 7 c.c. of normal saline; 5 c.c. of this suspension are made up to 50 c.c. with normal saline to form a 3 per cent. suspension of corpuscles as used in the Wassermann test. The remaining 5 c.c. of the mixture are added to 41 c.c. of distilled water; when lysis is complete, 4.2 c.c. of 10 per cent. sodium chloride are added, giving a strength of 0.85 per cent. salt in the whole amount; the lysed solution will then not produce any further lysis when mixed with the suspension of corpuscles. Mixtures are then made as indicated in Table IV; the last column shows the manner in which the terms in question were found to be employed.

Table IV.

*0.4 c.c. normal saline is placed in each tube to represent the volume of the other constituents present in a Wassermann test.*

Tube	Unlysed suspension c.c.	Lysed solution c.c.	Percentage of corpuscles lysed	Terms used to record degrees of lysis
1	0.4	0.1	20	Faint trace
2	0.35	0.15	30	
3	0.3	0.2	40	
4	0.25	0.25	50	Trace
5	0.2	0.3	60	
6	0.15	0.35	70	Distinct
7	0.1	0.4	80	
8	0.05	0.45	90	Very marked
9	0.025	0.475	95	Almost complete
10	0.02	0.48	96	
11	0.01	0.49	98	Just complete
12	0.005	0.495	99	
13	0.0	0.5	100	Complete

It is evident that the system given in this table is by no means ideal for statistical purposes. Firstly, from a quarter to a half of the corpuscles require to be lysed before the appearances which one would describe as "faint trace" or "trace" are produced; the point of commencing lysis cannot therefore be determined with much accuracy. Secondly, as it is much easier to estimate the smaller than the larger amounts of corpuscles, the later stages of lysis are over-represented; three of the seven percentages lie at or above 90. However, these defects seem unavoidable by any method which is practicable in testing large numbers of sera; and further, for the purpose of the present

investigation, the point of complete, or rather just complete, lysis is by far the most important, hence defective observation of the early stages of lysis is of no great significance.

## VI. DISCUSSION OF RESULTS.

The range of lysis observed with each serum was expressed graphically in the manner described above (p. 91); thus the amount of complement required to produce complete lysis with the negative control serum was reckoned as 100, and the amount required with each of the other sera to produce the whole range of lysis observed was expressed upon the same scale. The result is shown in Figs. 2 and 3. (1) The sera are plotted from left to right in the order of magnitude of the amounts of complement which they require in order to give complete lysis, that is, in the order of height reached by the vertical lines. (2) When lysis is complete in the first tube, the serum in question is represented by a dot only, placed at the level corresponding to the amount of complement in this tube. (3) A horizontal line is drawn across each figure at the level 100, which represents complete lysis with the negative control serum. Dots falling upon this line are not represented, but the length of this line between 50 and 100 is of course proportional to the number of these dots; there would be 199 dots on this line in Fig. 2, and 91 in Fig. 3. (4) A "faint trace" or "trace" of lysis has unavoidably to be taken as indicating the actual starting point of the process, though this is of course not accurate (see Table IV). (5) When the degree of lysis in the first tube is more than a trace, but is still incomplete, the line representing the serum bears an arrow directed downwards, to indicate that no point of initial lysis could be observed. (6) Sera with which lysis is still incomplete in the last tube, that is, with the largest amount of complement employed, are excluded from the graph, since it is the position of the point of complete lysis which is the basis of classification. The latter sera are all undoubtedly positive, and their removal does not affect the consideration of the doubtful zone. The negative control sera of each of the 92 days' tests are also excluded. By this elimination, the number in Series I is reduced from 540 to 408 and in Series II from 533 to 352.

In considering the results presented in the graphs Figs. 2 and 3, the sera were divided into groups, each increase of 50 per cent. in the amount of complement required to produce complete lysis constituting a group. Thus Group I shows complete lysis with not more than 50 per cent. of the amount of complement giving complete lysis with the negative control serum; Group II shows complete lysis with 51 to 100 per cent. of this amount, and so on up to Group X which extends from 451 to 500 per cent. The limits of each group are marked upon the horizontal line at the top of each figure. The length of each group was then measured, and these lengths calculated as percentages of the total length of the series; the distribution of the sera was thus ascertained, and is shown in numerical form in Table V.

It is at once evident upon inspection of Figs. 2 and 3 that no very abrupt



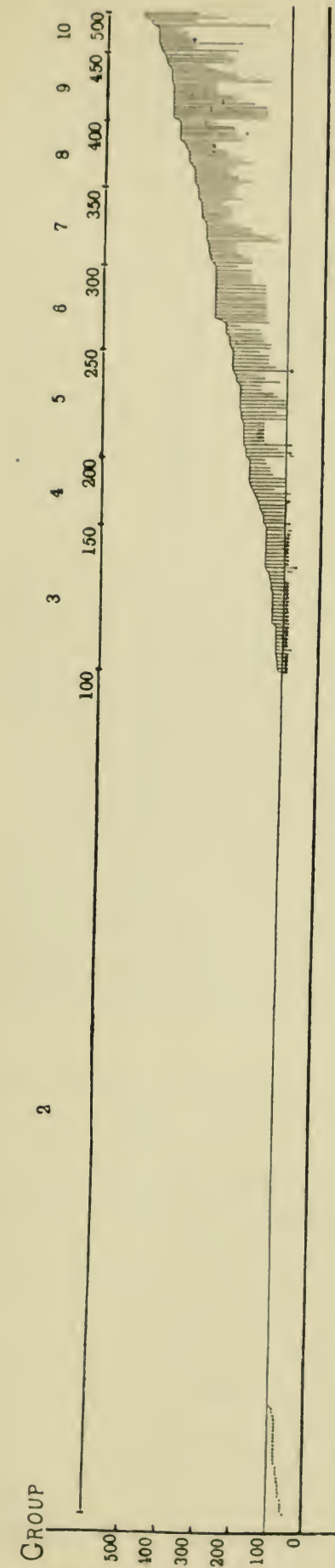


Fig. 2. Series I.

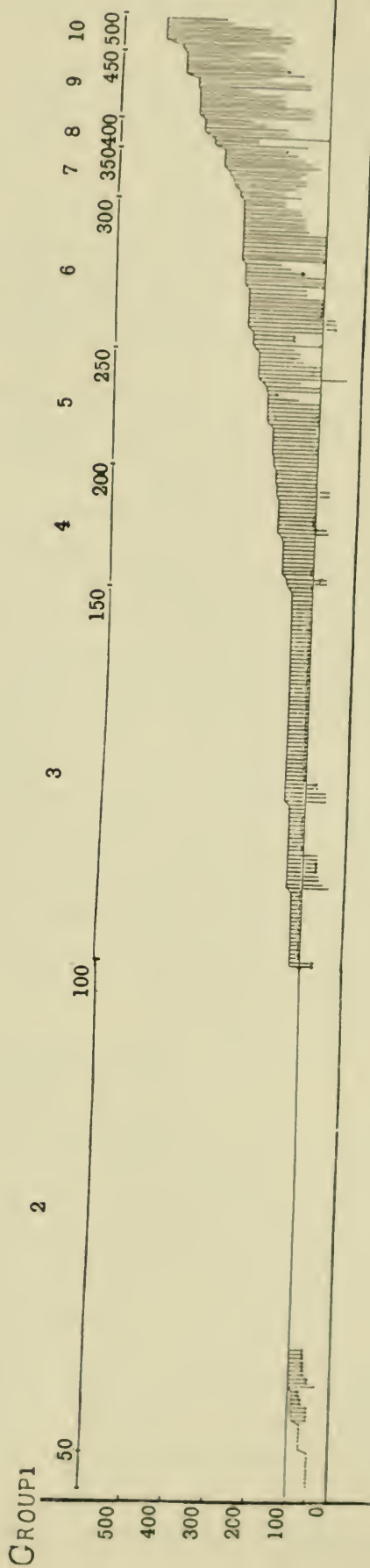


Fig. 3. Series II.

Table V.

Numerical results from all sera represented in Figs. 2 and 3.

Amount of complement required to produce com- plete lysis with negative control serum = 100		Series I			Series II			Series I and II combined		
		Total	per cent.	Total of per- centages	Total	per cent.	Total of per- centages	Total	per cent.	Total of per- centages
Group	Lysis complete at or below 50	0	—	—	9	2.5	—	9	1.2	
I										
II	51-100	229	56.1	—	116	32.8	35.3	345	45.4	46.6
III	101-150	40	9.8	65.9	91	25.9	61.2	131	17.2	63.8
IV	151-200	18	4.4	70.3	29	8.2	69.4	47	6.2	70.0
V	201-250	29	7.2	77.5	28	8.0	77.4	57	7.5	77.5
VI	251-300	23	5.6	83.1	36	10.3	87.7	59	7.8	85.3
VII	301-350	21	5.1	88.2	12	3.4	91.1	33	4.3	89.6
VIII	351-400	19	4.7	92.9	7	2.0	93.1	26	3.4	93.0
IX	401-450	18	4.4	97.3	16	4.6	97.7	34	4.5	97.5
X	451-500	11	2.7	100.0	8	2.3	100.0	19	2.5	100.0
Total ...		408			352			760		

change or discontinuity occurs. Hence the distinction between positive and negative sera is not capable of being very sharply defined. This is what would be expected by anyone who has experience of the Wassermann test when carried out by a quantitative method. But in both graphs a definite steepening of the gradient appears at the beginning of Group IV (151 to 200); in Table V this is represented by a fall in the percentage distribution, namely, from 9.8 to 4.4 in Series I, from 25.9 to 8.2 in Series II, and from 17.2 to 6.2 in the combined series. If the strongest positives, the omission of which from the graphs was mentioned above (p. 96), were included the fall in the percentage would be from 8.2 to 3.7 in Series I, and from 18.5 to 6.1 in Series II. The evidence, therefore, points to this region as the one in which is to be found the change in character of the sera from negative to positive. In the light of this result, we would propose that the following system should be applied in diagnosis:

- (1) Sera which allow of complete lysis with not more than 150 per cent. of the amount of complement giving complete lysis with the negative control serum are negative (Groups I, II, and III).
- (2) Sera which do not allow of complete lysis with less than 200 per cent. of the amount of complement required by the negative control serum are positive (Group V and subsequent groups).
- (3) Sera falling between these two classes (from 150 to 200 per cent.) must be regarded as "doubtful" or "suspicious," i.e. Group IV.

It is to be noted also that the majority of those sera which fall into the next higher (200-250) and subsequent groups, give no lysis with 100 per cent. of complement, i.e. with the amount which causes complete lysis of the negative control. Accordingly, they would be reckoned as positive on the older one tube, "all or nothing," criterion and thus far the two methods of estimating a positive serum appear identical in their results. But, although



the sera of the 150–200 group show in many cases only commencing lysis at 100, the estimation of the amount of complement required to produce just *complete* lysis indicates that these sera are only very little removed from negative sera. This fact appears clearly when the sera of the 150–200 group are compared with those falling into the higher groups. Hence the latter test affords much more precise quantitative information as to the reacting power of the serum than does the one tube, “all or nothing,” criterion. On the other hand, certain sera which are quite definitely positive, since they require 250 per cent. or more of complement to give complete lysis, may show a very extensive range, with commencing lysis below 100 per cent. (see Fig. 3); the positive nature of such sera would appear questionable on the “all or nothing” basis.

It may be asked why the doubtful group should be arbitrarily limited to the 150 to 200 per cent. zone. In reply to this it may be said that the character of a serum which requires for complete lysis double the amount of complement required by the negative control, is definitely abnormal and, with the known exceptions, such abnormality indicates syphilis. But, as a further measure of precaution in the reckoning, those sera may be returned as *weak positives* which require for complete lysis not less than 200 nor more than 250 per cent. of complement. The rest, which number 87 per cent. of all those requiring 200 per cent. of complement or more for complete lysis, are frank *diagnostic positives* (see footnote, p. 103).

Confirmatory evidence of the value of the above criterion has been obtained from two sources, viz. (1) the examination of a large series of sera with heart-cholesterol antigen, the results of which have been computed by a somewhat different method (see Section VII (a)), and (2) a comparison of the results arrived at by the method used in Series I with the original diagnoses returned according to Browning and Mackenzie’s five dose criterion. (See Section VII (b).)

## VII. CONFIRMATORY EVIDENCE.

### (a) SERIES III.

As the method of calculation applied in Series I and II to over one thousand sera is somewhat laborious, an attempt was made to apply to a still larger number a method which, while consuming much less time, would extend the information as to the general distinction between positive and negative sera. Records of 1193 patients’ sera were employed, together with the corresponding 67 tests of a negative control serum derived from the same person as in the greater part of Series I. The human heart-cholesterol antigen was employed as described in Section IV. The percentages of lysis (see Table IV) shown in the three tubes containing a given serum were entered upon a card, and the cards arranged in the order of magnitude of these percentages. The results are summarised in Table VI.

The following points are exhibited by the table: (1) the negative control

serum showed 90 per cent. or more of lysis in the first tube on 66 out of 67 days; probably its appearance in Group IV on the remaining occasion was due to some error of technique. (2) With more than half the sera (57.6 per cent. comprising Groups I and II) the range of lysis in the first two tubes is identical with that shown by the negative control serum on the same or other occasions. Such sera cannot be regarded as other than negative and it is justifiable to conclude that they give complete lysis with at most 150 per cent. of the

Table VI.

*Results of the test of 1193 sera with heart-cholesterol antigen.*

The amount of complement in the first tube varied on different occasions from 1.3 to 3 M.H.D.; the second and third tubes always contained respectively 2 and 3 times as much complement as the first tube.

Relative amounts of complement ...		Percentage lysis in			Patients' sera			Patients known to have been treated		Number of times that negative control serum fell in group	
		1st tube	2nd tube	3rd tube	Number of sera	per- centage	Total of per- centages	No.	percentage of group		
		1	2	3							
Group	I	100	—	—	557	46.4	— 57.6	101	14.6	} = 14.8 per cent. of 1193	51
	II	90-98	100	—	134	11.2					15
	III	25-80	100	—	56	4.7	7.5	16	28.6		0
	IV	—	90-98	100	34	2.8		10	29.4		1
	V	—	0-80	100	21	1.75	66.8 69.1 76.3	18	13.2		
		—	—	90-98	28	2.3				34.3	
		—	—	25-80	87	7.25					
	VI	—	—	0	276	23.0	100	33	11.9		
Total...		—	—	—	1193	—	—	178	—		67

complement which causes complete lysis of the negative control. (3) The next two groups (III and IV) taken together show in the second tube the same range of lysis as is given by the negative control serum in the first. Since the ratio of the amounts of complement in the first, second, and third tubes was in all cases as 1 : 2 : 3 these sera (III and IV) taken all together require therefore twice as much complement as do the negative controls, also taken all together, to allow of a given degree of lysis. To compare in detail Groups III and IV with the negative control is a more difficult matter; for instance, the opinion to be formed of sera in Group III will depend upon whether, on the day in question, the negative control falls in Group I or Group II. The most satisfactory comparison is that between a serum in Group IV and the negative control in Group II; here one can state definitely that the respective amounts of complement required to produce 90 to 98 per cent. of lysis are as 1 : 2. A negative control in Group I is a less satisfactory basis for comparison, since one cannot tell whether or not the amount of complement present is in excess of that required to produce complete lysis.

It is noteworthy that of the cases in Groups III and IV the percentage which had undergone anti-syphilitic treatment, is twice as great as that



present in the whole series (roughly 28 per cent. and 14 per cent. respectively). The high proportion of treated cases in these groups is due to two factors: (1) in the course of cure, the serum of a person who has given a positive reaction must pass back through this doubtful zone, and (2) many persons are now treated before their serum has developed the full positive reaction; this will occur especially in clinics where the examination for spirochaetes is systematically carried out.

The general results derived from Series III are as follows:

(1) A large number of sera (57.6 per cent.) require for complete lysis not more than 150 per cent. of the complement which causes complete lysis with the negative control.

(2) A comparatively small number (7.5 per cent.) require from 150 to 200 per cent. of this amount. It is noteworthy that this group contains a greater proportion (double) of cases known to have been treated than any other.

(3) The remainder (34 per cent.) require more than 200 per cent. of complement. Two-thirds of these shows no lysis with 300 per cent. of the complement which lyses the negative control completely.

These results are therefore confirmatory of those given in the preceding section in the following points: (1) the frequency distribution of the sera shows an abrupt fall in the region corresponding roughly to Group IV of Series I and II, namely, where the amount of complement required to produce complete lysis reaches 150 to 200 per cent. of that required by the negative controls collectively. (2) This region, which it is proposed to regard as that of "suspicious" sera, shows a very high proportion of treated cases; and it is certain that such cases must always yield a large number of reactions which are doubtful on any system of diagnosis.

(b) COMPARISON OF THE PROPOSED METHOD OF DIAGNOSIS WITH THAT PREVIOUSLY USED.

When the sera included in Series I were submitted to the Wassermann test, now several years ago, the diagnoses were then made on the system stated on p. 88 above, under which a positive serum must require at least five doses of complement, in addition to the amounts inhibited by the serum and the antigen separately, to allow of complete lysis of the test corpuscles. One can now compare these original diagnoses with those suggested by the subsequent examination of the results which forms the subject of the present paper. This comparison is presented in Table VII and it will be seen that there is a close agreement between the diagnoses arrived at on the earlier and the later systems<sup>1</sup>.

The new data brought forward in this paper have suggested that (1) a serum in order to be counted as negative must allow complete lysis with

<sup>1</sup> Many of the diagnoses in Series II are not now available, so that the comparison in that Series cannot be made.

not more than 150 per cent. of the amount of complement required by the negative control; (2) a positive serum must require not less than 200 per cent. of this amount, and (3) sera in the intervening zone must be regarded as doubtful. Now it is seen in the table of original diagnoses that: (1) no

Table VII.

*Comparison of the proposed method of diagnosis with that previously used.*

Amounts of complement giving complete lysis (100 gives complete lysis with negative control)	Original diagnoses		
	Series I		
	-	?	+
50-100	229	0	0
101-125	12	1	0
126-140	11	3	0
141-150	9	4	0
151-175	0	5	1
176-200	0	4	8
201-225	0	3	9
226-250	0	0	186
251-275			
276-300			
301-325			
326-500			

negatives occur above 150; (2) the doubtful cases, though extending below 150 and above 200, attain their maximum between these two points; (3) there is only one positive below 175; (4) above 225, all were diagnosed as positive.

The original "5 dose rule" was arrived at by the combined clinical and laboratory examination of a large number of cases; hence the fact that it gives diagnoses in general agreement with those which would be made on the new system put forward here provides valuable support for the latter. Discrepancies in the case of sera in the doubtful zone are bound to occur when such results, obtained on different days with complements of varying deviability, are pooled. Thus, nine sera were diagnosed as positive which would be considered doubtful by the new rule; on the other hand, three sera originally returned as doubtful would now be called positive. The discrepancies, therefore, amount to only 2.5 per cent. and do not involve any transference from the negative to the positive category or *vice versa*. In the face of this difficulty, the agreement between the two systems seems to be quite as close as can be expected. Of course, the delimitation of the "doubtful" region must always be a difficult and wholly arbitrary matter.

VIII. THE APPLICATION OF THE RESULTS IN DIAGNOSIS.

In practice a compromise has to be made between (1) the large number of tubes which is needed to show adequately the range of lysis with each serum, and (2) the small number of tubes which is practicable in routine testing on a large scale. Latterly we have used three tubes (exclusive of the serum control) containing amounts of complement in the ratio of 1 : 2 : 3;



these amounts being such as to give 2, 4, and 6 M.H.D. in accordance with the preliminary estimation of the dose (see note I at the end of this paper). With this arrangement the great majority of sera will fall into two classes, namely, (*A*) negatives, showing in the first tube the same, or nearly the same, degree of lysis as does the negative control in this tube; and (*B*) positives, showing in the second tube either nearly the same degree of lysis as does the negative control in the first (weak positives), or less than this (diagnostic positives)<sup>1</sup>; i.e. the positive sera deviate not less than twice as much complement as does the negative control. For the diagnosis of the weakest positives, it is very desirable that the negative control should not show complete lysis in the first tube, so that an exact comparison can be made. It is, of course, the sera intermediate between the classes (*A*) and (*B*) above which cause difficulty. In the series of tubes described above there is no provision for exact observation of the "doubtful" class which requires from 150 to 200 per cent. of the amount of complement required by the negative control; on this account it seems that 2, 3, and 4 doses of complement would be preferable to the 2, 4, and 6 doses used in the work which led subsequently to these conclusions. It would certainly be advisable to use the 2, 3, 4 dose range when retesting suspicious sera. When 2, 4 and 6 doses are used, and one requires to pick out the 150 per cent. group of sera, one has to assume

Table VIII.

*Scheme of diagnosis of negative, suspicious and weak positive sera.*

		Percentage of lysis			Diagnosis
Complement M.H.D.		Tube 1	2	3	
		Ratio 1	: 2	: 3	
Type I	Known negative	100	—	—	(Control)
	Patients' sera	60-70	100	—	Negative (border line)
		50 or less	100	—	Suspicious
		20-80	95-98	100	Weak positive (lowest limit)
Type II	Known negative	90	100	—	(Control)
	Patients' sera	40 or more	100	—	Negative (border line)
		Less than 40	More than 90	100	Suspicious
		Less than 40	Less than 90	100	Weak positive (lowest limit)

*Note 1.* The range of lysis given for the negative control is the same as that observed on 66 occasions in Series III (Table VI).

2. Very abrupt increase of lysis (e.g. faint trace in one tube and complete in the next) is suggestive of possible errors in technique, as is likewise a very gradual increase (e.g. very marked, almost complete, just complete, in the three tubes) and it is well to retest such sera, though some will be found to act constantly in this way.

<sup>1</sup> It is to be understood that there are two criteria of positive: firstly the absolute positive which is required for diagnosis in an unknown case, and to which one would swear in a court of law, and secondly the weak positive or suspicious reaction, which is as good as positive in a case of known treated syphilis or in one which had reacted positive prior to treatment; the latter reaction might be termed the *therapeutic* positive in contra-distinction to the former, the *diagnostic* positive.

that a serum which requires 150 per cent., or  $3/2$  of the complement required by the negative control, should theoretically show  $2/3$  or 66 per cent. lysis (i.e. distinct) with the amount which gives complete lysis with the negative control. A general scheme of diagnosis suggested by these considerations is given in Table VIII (p. 103). It is, of course, impossible to state any such method in a form which can be applied mechanically in all cases. In proportion as the negative control serum deviates more or less complement on any particular day, so must the classification of patients' sera be shifted in one or the other direction; the table is intended to provide a basis for judging of the results in this way.

#### USE OF A POSITIVE CONTROL.

In addition to the negative control, a positive control serum should, of course, always be included in each series; the latter should be comparatively weak, giving complete lysis with the highest amount of complement used. When the same positive control is employed on repeated occasions one obtains a check upon the deviability of the complement; thus if the positive control on a given day requires less complement than usual to give complete lysis, one knows that a less deviable complement has been used and therefore that more significance attaches to suspicious or weakly positive results and *vice versa*.

#### THE SERUM CONTROL.

The action of each patient's serum on complement when salt solution is substituted for the "antigen" should never be omitted, since occasionally human sera are met with which are abnormally inhibitory toward complement (see Kennaway and Wright). Any serum which, along with two doses of complement, gives less than almost complete lysis, is abnormal in this respect and should be retested, since, of course, such inhibitory action will vitiate the result.

#### SUMMARY.

Examination of the records of a large number of quantitative Wassermann tests has suggested a new and simple criterion of a positive reaction. This is based upon a comparison of the amounts of complement giving complete lysis with any given serum, and with the negative control, respectively. Reasons are given for regarding this criterion as more satisfactory than that in common use. Details are given of the methods employed for the selection of a negative control serum, the estimation of degrees of lysis, and the provision of a suitable range of amounts of complement.



NOTE I.

THE AMOUNTS OF COMPLEMENT USED IN WASSERMANN TESTS.

The consideration of the results of Wassermann tests is facilitated if the different amounts of complement used bear a simple ratio to one another and it is preferable that these amounts should contain whole numbers of haemolytic doses; the latter arrangement is especially to be recommended if the results are to be used for statistical purposes. Unfortunately the haemolytic dose as estimated in the course of the actual test of the sera (complement control) is not always the same as that found in the preliminary test of the complement, in the light of which the amounts to be used in the actual test are selected; hence adherence to any system of dosage sometimes breaks down in practice. However, the following scheme has been found useful (Table IX).

Table IX.

*Number of Haemolytic Doses to be used (for three tubes of antigen plus patient's serum).*

Amounts of comple- ment giving number of doses required	Haemolytic dose (for 0.5 c.c. of 3 per cent. red corpuscle suspension sensitised with 5 doses of immune body)			
	0.0025 c.c.	0.005 c.c.	0.0075 c.c.	0.01 c.c.
·01 c.c.	4 doses	2 doses		
·015 „	—	—	2 doses	
·02 „	8 „	4 „	—	2 doses
·03 „	12 „	6 „	4 „	
·04 „	—	—	—	4 „
·045 „	—	—	6 „	
·06 „	—	—	—	6 „

NOTE II.

PROPORTIONS OF POSITIVE AND NEGATIVE SERA.

It is of interest to note the proportions of negative and positive sera observed in ordinary hospital practice, as shown in Table X. In Series I and IV the liver-*lecithin-cholesterol* antigen was used and in Series III heart

Table X.

Results (per cent.)		Liver- <i>lecithin-cholesterol</i> antigen			Heart- <i>cholesterol</i> antigen
		Series I (485 sera)	Series IV <sup>1</sup> (508 sera)	Series I and IV combined (993 sera)	Series III (1193 sera)
Negative	...	53.8	53.6	53.8	59.6
Suspicious	...	4.1	7.0	5.6	2.7
Weak positive	...	2.6	2.9	2.8	2.3
Positive	...	39.5	36.5	37.8	35.4
		100.0	100.0	100.0	100.0

<sup>1</sup> The analysis of Series IV in the manner described in this paper was not carried out owing to the number of occasions on which the point of just complete lysis with the negative control serum could not be observed.

extract plus cholesterol. The strongest positives of Series I, which were omitted from Fig. 2 (see p. 96), are of course included here.

The heart-cholesterol antigen appears to give the slightly smaller number of suspicious results, but the number of weak positives is the same with both antigens. An unqualified return of "negative" or "positive" was made in 92-95 per cent. of the cases.

## REFERENCES.

- BOAS (1914). *Die Wassermannsche Reaktion*. Berlin.  
BROWNING, CRUICKSHANK and MACKENZIE (1910). *Journ. of Path. and Bact.* xiv. 484.  
—— and KENNAWAY (1919). *Lancet*, i. 785.  
—— and MACKENZIE (1909). *Zeitschr. f. Immunitätsforsch.* ii. 459.  
—— (1911). *Recent Advances in the Diagnosis and Treatment of Syphilis*. London.  
FILDES and MCINTOSH (1913). *Brain*, xxxvi. 193.  
—— (1913). *Medical Research Committee, Special Report Series*, No. 14.  
KENNAWAY and WRIGHT (1919). *Journ. of Hygiene*, xviii. 255.  
WASSERMANN (1913). See WASSERMANN and LANGE. *Handbuch d. Pathogen. Mikroorganismen* (Kolle-Wassermann), 2 Ed. Berlin, vii. 951.



## THE MILK SUPPLY OF THE CITY OF EDINBURGH.

BY ANDREW CUNNINGHAM, B.Sc. AND B. A. THORPE.

(*Bacteriology Dept., Edinburgh and East of Scotland College of Agriculture.*)

THE objects of the investigations described in this paper were:

- (1) To obtain data on the bacteriological quality of the milk supply of the city of Edinburgh;
- (2) To compare such data with similar information for other cities;
- (3) To demonstrate the value of certain biochemical tests for milk, at present little used in this country.

In the past a large amount of investigational work has been carried out along the general lines of the present enquiry. But frequently too much stress has been laid on the total amount of the bacterial contamination of milk and too little attention paid to its quality. A milk containing several hundreds of intestinal lactose-fermenting organisms per c.c. is much more likely to have injurious effects on the consumer than one containing several millions of normal lactic acid bacteria in the same quantity. Thus, it is of considerable importance to have a method for the examination of milk which will rapidly and fairly accurately demonstrate the nature of the bacterial contamination. The milk fermentation test, which will be described in this paper, fulfils these requirements.

In milk investigations and other work account must also be taken of the quantity of bacterial contamination. This is generally determined by plating known dilutions on ordinary or whey agar. Results are not obtained much inside a week and a considerable amount of special apparatus is required. The reductase test from which results can be got in a few hours is much more convenient and gives sufficiently reliable indications of the quantity of bacterial contamination.

### METHODS.

*Sampling.* The samples used in this work were taken in small sterile milk cans. The can was taken to the milk shop and the milk bought under the same conditions as applied to sales to the general public. The samples were immediately taken to the Laboratory and examined.

The following tests were carried out for each sample:

- (1) *Determination of Bacterial Content.* Counts were made in the usual way by the dilution method on whey agar at 22° C. (incubated for 10 days). Powdered chalk was added to the medium before it solidified to distinguish acid-producing colonies. The acid formed by the latter dissolves the chalk

in their neighbourhood: thus acid-forming colonies are surrounded by a clear zone in the otherwise turbid medium.

(2) *Determination of Content of Lactose Fermenters.* Known dilutions of the milk were inoculated into bouillon containing 1 % lactose in test tubes, each containing a fermentation tube. The bouillon was incubated at 37° C. for three days and the presence or absence of gas formation in the various dilutions noted.

(3) *Reductase Test.* This test depends on the power, possessed by all bacteria, of reducing and decolourising solutions of certain stains. Thus the higher the bacterial content of a milk sample the more quickly does it decolourise the stain. 40 c.c. of the milk sample were taken in a sterile boiling-tube and 1 c.c. dilute methylene blue solution (5 c.c. saturated alcoholic solution stain + 195 c.c. distilled water) added. The tube was incubated at 37° C. and the time required for the decolourisation of the stain noted.

(4) *Fermentation Test.* 40 c.c. of the milk sample were placed in a sterile boiling-tube and incubated for 24 hours at 37° C. The resulting curd is a good guide to the nature of the bacterial contamination. The following are the chief types:

(a) Milk remains liquid: contains few bacteria, generally only micrococci from the inside of the cow's udder, which have little action on milk.

(b) Curd gelatinous and uniform with no whey: sample contains chiefly true lactic acid organisms.

(c) Curd gritty: sample contains considerable numbers of true lactic acid bacteria and also sporing rods from dirty milk vessels, dust, air, etc.

(d) Curd cheesy: sample contains mainly sporing rods.

(e) Curd blown: abundant gas formation: milk contains large numbers of intestinal, lactose-fermenting organisms.

A good quality milk should give a curd of type (a) or (b): i.e. it should contain only organisms to be regarded as normal to milk. Contaminated milk will give a gritty, cheesy or blown curd. The last named is to be regarded as the worst: next comes the cheesy type, followed by the gritty which may be only slightly inferior to the gelatinous.

(5) *Catalase Test.* Milk possesses the power of liberating oxygen from hydrogen peroxide. The constituents of milk responsible for this change are the bacteria and enzymes and the animal cells derived from the inside of the cow's udder. But all the bacteria in milk cannot liberate oxygen from hydrogen peroxide: the most notable exceptions are the true lactic acid bacteria. 10 c.c. of the milk sample were mixed with 5 c.c. hydrogen peroxide (1 %) and the gas evolved during 24 hours at 37° C. measured. Koestler's apparatus was employed.

(6) *Sediment Test.* 10 c.c. of milk were placed in a centrifuge tube and warmed to 60° C. The tube was then centrifuged for 5 minutes. Observations were made on the quantity, colour and nature of the sediment from which a film was made and stained with aqueous methylene blue.



## DISCUSSION OF RESULTS.

The results obtained from the examination of 40 samples of Edinburgh milk are shown in Table I.

Table I.

No. of sample	Bacteria per c.c.	Acid producers		Non-acid producers		Reduced in hrs.	Lactose Fermenters		Fermentation test		Catalase c.c. oxygen
		per c.c.	%	per c.c.	%		per c.c.	%	Gas formation	Type of curd	
1	270,000	130,000	48	140,000	52	>5½	500	·185	g*	Sl. gritty	—
2	350,000	20,000	6	330,000	94	>5½	1	·000	—	Liquid	1·7
3	100,000	35,000	35	65,000	65	>5½	500	·500	—	Sl. gritty	2·4
4	29,000,000	26,000,000	90	3,000,000	10	4	10,000	·034	B†	—	2·5
5	20,000	<10,000	<50	>10,000	>50	>5½	500	2·500	B	—	1·9
6	280,000	230,000	82	50,000	18	>5½	100	·036	B	—	2·5
7	160,000	50,000	31	110,000	69	4	5,000	3·125	B	—	3·2
8	120,000	50,000	42	70,000	58	>5½	10	·008	—	Cheesey	1·9
9	310,000	280,000	90	30,000	10	>5½	10	·003	—	Gritty	2·1
10	130,000	30,000	23	100,000	77	>5½	10	·008	g	Cheesey	1·9
11	90,000	30,000	33	60,000	67	>5½	10	·011	B	—	2·4
12	3,300,000	160,000	5	3,140,000	95	4½	100	·003	g	Cheesey	2·1
13	280,000	70,000	25	210,000	75	>5½	100	·036	g	„	3·2
14	230,000	150,000	65	80,000	35	>5½	10	·004	—	Gritty	1·9
15	800,000	500,000	62	300,000	38	>5½	100	·012	—	„	2·6
16	165,000	80,000	48	85,000	52	>5½	10	·006	—	„	1·9
17	210,000	70,000	33	140,000	67	5½	100	·048	g	„	2·1
18	600,000	90,000	15	510,000	85	>5½	10	·002	g	Cheesey	1·2
19	320,000	70,000	22	250,000	78	4	1,000	·312	—	„	2·0
20	160,000	40,000	25	120,000	75	>5½	100	·063	B	—	1·5
21	170,000	60,000	35	110,000	65	>5½	1	·000	—	Gritty	1·9
22	60,000	15,000	25	45,000	75	>5½	10	·017	—	Cheesey	1·4
23	600,000	350,000	58	250,000	42	>5½	—	·000	—	Sl. gritty	3·7
24	390,000	140,000	36	250,000	64	>5½	1	·000	—	Cheesey	2·25
25	3,050,000	1,000,000	33	2,050,000	67	3¼	100	·003	g	„	4·8
26	1,500,000	180,000	12	1,320,000	88	4	1,000	·067	B	—	4·5
27	960,000	400,000	42	560,000	58	>5½	10	·001	—	Cheesey	2·1
28	280,000	140,000	50	140,000	50	>5½	100	·036	B	—	1·5
29	29,000,000	1,000,000	3	28,000,000	97	2¾	10	·000	—	Cheesey	2·6
30	420,000	190,000	45	230,000	55	>5½	1,000	·238	B	—	1·2
31	450,000	250,000	56	200,000	44	5	1,000	·222	B	—	2·1
32	9,300,000	9,200,000	99	100,000	1	4	1,000	·011	—	Sl. gritty	3·9
33	12,500,000	7,500,000	60	5,000,000	40	3¼	10	·000	—	Gritty	2·1
34	120,000	35,000	29	85,000	71	>5½	100	·083	—	Sl. gritty	1·7
35	1,250,000	700,000	56	550,000	44	>5½	10	·000	—	„	4·6
36	450,000	200,000	56	250,000	44	>5½	50	·011	—	„	5·0
37	155,000	130,000	84	25,000	16	5	—	·000	B	—	6·3
38	160,000	70,000	44	90,000	56	>5½	—	·000	g	Cheesey	4·6
39	125,000	15,000	12	110,000	88	>5½	—	·000	—	„	6·4
40	60,000	500	1	59,500	99	>5½	1	·002	—	„	3·9

\* g = slight gas formation.

† B = blown curd.

*Bacterial Counts.* The bacterial content of the samples examined varied from 20,000 to 29,000,000 per c.c. or just over 2,500,000 on the average. But an average of figures varying so widely does not convey much: the few samples with a content of over 1,000,000 were in many cases badly con-

taminated and so greatly raised the average. A better and fairer idea of the extent of the bacterial contamination is got by classifying the counts. Four of the samples (10 %) contained fewer than 100,000 organisms per c.c.; 24 (60 %) gave counts varying from 100,000 to 500,000; four (10 %) gave results between 500,000 and 1,000,000 and eight samples (20 %) showed more than 1,000,000 bacteria per c.c. Thus the majority of the samples show counts varying from 100,000 to 500,000 per c.c.

These figures, when compared with those for other cities, do not appear to be abnormally large—though they could be greatly lowered by the introduction of better methods of production and handling of milk. Table II (1) shows

Table II.

Year	City	Bacteria per c.c.
1885	Amsterdam	2,500,000– 10,500,000
1889	Munich	200,000– 6,000,000
1889	Würzburg	1,000,000– 2,000,000
1891	Halle	6,000,000– 30,000,000
1892	Giessen	83,100–169,632,000
1892	Würzburg	1,200,000– 7,200,000
1893	Dorpat	2,000,000–117,000,000
1894	Christiania	160,000– 45,000,000
1895	Petrograd	400,000–115,300,000
1895	Middletown	11,000– 8,500,000
1898	Guelph	121,000– 1,200,000
1898	Königsberg	12,500– 21,500,000
1899	Helsingfors	20,000– 34,300,000
1900	Dresden	250,000– 5,478,000
1901	New York	250,000– 30,000,000
1904	Middletown	8,000– 3,000,000
1905	Madison	35,000– 2,000,000
1905	Raleigh	34,000– 6,768,000
1905	Moscow	100,000– 7,000,000
1907	Raleigh	1,200– 54,000,000
1907	London	20,000– 8,000,000
1907	Berlin	43,000– 11,500,000
1907	Munich	204,000– 4,251,000
1907	Budapest	52,800– 18,000,000
1909	Chicago	10,000– 18,000,000

corresponding data for 25 other cities—from results published between 1885 and 1909. Since that date the main figures published are summarised below :

Table III.

Year	City	Bacteria per c.c.	Authority*
1910	Copenhagen	400,000– 32,000,000	(2)
1910	Lisbon	73,000–271,000,000	(3)
1911	St Paul, Minn.	Average = 8,206,000	(4)
1911	Chicago	10,000–100,000,000	(5)
1912	Leipzig	27,500– 142,000,000	(6)
1912	Washington	Under 500,000 = 35%: over 500,000 = 65%	(7)
1912	New York	„ 100,000 = 83%: over 100,000 = 17%	(8)
1913	Moscow	13,000– 82,500,000	(9)
1917	Geneva, N.Y.	Under 200,000 = 88%: over 200,000 = 12%	(10)

\* See numbered references at end of this Paper.



It will be seen that the figures obtained here compare quite well with those got for other cities—with the exception of New York where the milk supply is particularly clean. But it is well to bear in mind that many of the figures quoted apply to samples taken throughout the whole year, whereas, in the present enquiry, we have dealt exclusively with milks obtained during the winter months. The low winter temperature would naturally favour low bacterial counts.

But though the figures obtained compare well with those for other cities it must not be supposed that the Edinburgh milk supply is particularly clean. As will be shown later the nature of the bacterial contamination is particularly bad. And, in addition, great reductions in total bacterial numbers will have to be made before the supply can be considered a really clean one.

*Reductase Test.* The standards suggested by Barthel and Jensen<sup>(11)</sup> for use with this test are as follows:

Quality of milk	Time required for reduction	Bacteria per c.c.
Good	Longer than $5\frac{1}{2}$ hrs.	About 500,000
Medium	$2-5\frac{1}{2}$ hrs.	500,000–4,000,000
Poor	20 mins.–2 hrs.	4,000,000–20,000,000
Very poor	Under 20 mins.	Over 20,000,000

According to these standards 28 of our samples (70 %) would be classed as good and the remaining 12 (30 %) as medium. These figures correspond well with those given under “Bacterial Counts” where it is shown that 70 % of the samples gave counts under 500,000.

*Relation between Bacterial Counts and Reductase Test.* The time required for reduction corresponds fairly well with the bacterial counts except in a very few cases. Those samples which contained large numbers of bacteria have, in practically every case, shown a short reduction period: while generally where the numbers of bacteria present have been small the time required for reduction has been fairly long. But the bacterial counts and times of reduction do not correspond well with those laid down by Barthel and Jensen<sup>(11)</sup> (see above) and, as has been noted by Schroeter<sup>(6)</sup>, the latter would appear to require some modification. Our results lead us to the conclusion that a milk sample which reduces within  $5\frac{1}{2}$  hours generally contains 1,000,000 or more bacteria per c.c. The only exceptions in the present investigation were samples 7, 19, 31, 35 and 37. The only case in which the reductase test failed to show a milk which contained more than 1,000,000 organisms per c.c. was sample 35, and here the bacterial content was just over 1,000,000 and the sample was otherwise a comparatively clean one (contained fewer than 0.001 % of lactose fermenters). In the four cases in which the reductase test showed a short reduction period for samples containing fewer than 1,000,000 organisms per c.c., all are to be regarded as badly contaminated samples. Nos. 7, 19 and 31 contained a high percentage of lactose fermenters: No. 37 produced a blown curd in the fermentation test. It may be said, therefore, that in no

case has the reductase test condemned a really clean sample and with one exception—and that an unimportant one—it has never failed to detect a sample containing more than 1,000,000 bacteria per c.c. when the standard is modified as above suggested.

*Content of Lactose Fermenters.* The content of lactose fermenters varied widely (from 0 to 10,000 per c.c.). 20 % of the samples contained lactose fermenting bacteria in numbers of 1 or under per c.c. of the milk; 30 % showed these organisms in numbers from 1 to 10 per c.c.; 25 % in numbers from 10 to 100 per c.c.; 20 % in numbers from 100 to 1000 per c.c.; 5 % in numbers from 1000 to 10,000 per c.c.

Various standards have been suggested for the numbers of lactose fermenters in clean milk. Vanderleek<sup>(12)</sup> holds that they should not constitute more than 1 % of the total bacterial count—a standard which appears to err on the side of leniency (cf. Schroeter). Savage<sup>(13)</sup> considers that in winter milk might be classified as follows with regard to its content of lactose fermenters:

Pass = under 100 per c.c.; Unsatisfactory = 100–500 per c.c.; Condemn = over 500 per c.c. Kinyoun<sup>(14)</sup> found that the average for good milk was 1 lactose fermenter per 50,000 other bacteria ( $\cdot 002$  %) while dirty milk contained 1 lactose fermenter per 555 others ( $\cdot 180$  %). Ayers, Cook and Clemmer<sup>(15)</sup> found lactose fermenting organisms in  $\cdot 01$  c.c. of milk in a relatively small number of samples.

From the data given by Savage and by Ayers, Cook and Clemmer it seems not unreasonable to expect that in clean milk not more than 10 lactose fermenters per c.c. should be found. In the samples examined by us which showed lactose fermenters in numbers up to 10 per c.c. none gave a percentage of these organisms in the total count higher than  $\cdot 02$ —a figure which corresponds well with the data given by Kinyoun.

If one takes the standard of 10 lactose fermenters per c.c. it is found that 20 samples (50 %) would be condemned. Taking the standard as  $\cdot 02$  % of the total count, 15 samples (37.5 %) fail to satisfy the requirement. Both of these percentages are abnormally high and indicate either very considerable original contamination with undesirable organisms or great multiplication of these organisms in the milk on keeping. But the organisms of the intestinal group require a high temperature for their activity—somewhere in the neighbourhood of  $37^{\circ}$  C. And as the air temperature during the period covered by these experiments was very low and the samples were generally found to be well cooled in the retailers' premises, one is forced to the conclusion that the responsibility for the high counts rests mainly on the producer. The high figures for lactose fermenters would appear to be due to insufficient attention to cleanliness in the production of the milk.

*Fermentation Test.* The results of this test are given in two columns headed "Gas formation" and "Type of curd." In the former only those samples which produced gas are noted: in the latter those which did not produce blown curds. Eleven samples (27.5 %) gave blown curds while eight



(20 %) produced small quantities of gas in the test. These results are far from satisfactory and support those of the counts of lactose fermenters in emphasising the undesirable nature of the bacterial contamination. They show that about half of the samples contain lactose fermenters in dangerously high numbers.

There is no very marked agreement between the formation of gas in the fermentation test and the number of lactose fermenters per c.c. or the percentage of the latter in the total bacterial count. Generally, however, the badly contaminated milks have produced blown curds. Further the test is probably a fairer one both from the public health and dairying points of view than simple counts of the lactose fermenters because:

- (i) a large quantity of milk is examined (40 c.c.),
- (ii) it is carried out in the milk itself.

In those cases where the gas-forming organisms have taken the upper hand in the fermentation test they probably would have done so if the milk had been consumed or if it had been made into cheese.

Of the 29 samples which did not show a blown curd in the fermentation test only one remained liquid: 14 gave gritty curds and 14 cheesy curds. The sample which remained liquid (No. 2) contained a very low percentage of acid-producing organisms and the non-acid producers present evidently had little action on milk. None of the samples examined gave an absolutely uniform gelatinous curd. Thus according to the results of the fermentation test only one sample could be considered to be really first class.

Milks which produce a gritty curd are to be regarded as fairly satisfactory. The proportion (35 %) in this case is decidedly low, however, when one remembers that only one sample can be classed in types 1 and 2. In the gritty samples the percentage of non-acid producers varies from 1 to 71.

Samples which give a cheesy curd are generally highly contaminated with spore-bearing organisms from fodder, soil, air, improperly sterilised milk vessels, etc. 35 % of the samples showed cheesy curds. These samples contained 56–99 % of non-acid producing organisms.

Thus of the 40 samples examined, 14 produced cheesy and 11 blown curds. Both types are unsatisfactory and indicate high contamination. Together they make up 62·5 % of the samples investigated—a proportion which is unduly high.

*Catalase Test.* The figures for the catalase test vary widely and no relation could be found between them and the other results.

*Sediment Test.* The amount of sediment present in the samples only exceeded ·1 % in one case (No. 3). Generally the sediment was brownish in colour and streptococci, rods and cells could frequently be seen in it.

## SUMMARY AND CONCLUSIONS.

In tests of 40 samples of milk from the supply of the city of Edinburgh:

(1) The bacterial content was, on the average, high but not abnormally so when compared with that of the milk supply of other cities. It ranged from 20,000 to 29,000,000 per c.c.

(2) The results of the reductase test corresponded well with the bacterial counts and the test is recommended for obtaining a good rough idea of the bacterial numbers present, where results are required in a short time.

(3) About half of the samples contained lactose-fermenting organisms in unduly high numbers; this is attributed chiefly to the lack of attention to cleanliness in production.

(4) The value of the fermentation test for speedily indicating the nature of bacterial contamination is pointed out. In a general way the test corroborated the results of counts of the lactose fermenters and indicated that a number of the samples, which did not produce blown curds, contained large numbers of undesirable organisms.

(5) Taken as a whole, the results seem to indicate not so much particularly high contamination as contamination of a very undesirable nature. They point to the necessity of educating the producer and distributor in the principles underlying the production of clean milk and emphasise the importance of proper control of the industry from this point of view.

## REFERENCES.

- (1) LÖHNIS, F. (1910). *Handbuch d. landw. Bakt.* 156.
- (2) JENSEN, O. (1910). *Rev. Gén. du Lait*, VIII. 49-60; abstr. in *Expt. Sta. Rec.* (1910), 478.
- (3) BORGES, I. and FERREIRA, A. A. (1910). *Arch. R. Inst. Bact. Camara Pestana*, III. 99-135; abstr. in *Expt. Sta. Rec.* 23 (1910), 179.
- (4) KELLY, E. (1911). *U.S. Dept. Agr. Bur. Anim. Indus. Rpt.* 237-46; abstr. in *Expt. Sta. Rec.* 29 (1913), 776.
- (5) KOEHLER, G. and TONNEY, F. O. (1911). *Journ. Amer. Med. Assoc.* LVI. 713-8.
- (6) SCHROETER, O. (1912). *Centralbl. f. Bakt.* II. Abt. XXXII. 181-92.
- (7) KINYOUN, J. J. and DEITER, L. V. (1912). *Amer. Journ. Pub. Health*, II. 262-74; abstr. in *Expt. Sta. Rec.* 27 (1912), 281.
- (8) SCHROEDER, M. C. (1912). *Journ. Infect. Dis.* XI. 1-20; abstr. in *Expt. Sta. Rec.* 27 (1912), 879.
- (9) WOJTKIEWICZ, A. (1913). *Centralbl. f. Bakt.* II. Abt. XXXIX. 53-61.
- (10) BREED, R. S. and BREW, J. D. (1917). *New York State Sta. Bull.* No. 443, 717-46; abstr. in *Expt. Sta. Rec.* 39 (1918), 4, 383.
- (11) BARTHEL, C. and JENSEN, O. (1912). *Milchwirtsch. Centralbl.* XLI. 417-29; abstr. in *Expt. Sta. Rec.* 29 (1913), 206.
- (12) VANDERLECK (1909). *Centralbl. f. Bakt.* II. Abt. XXIII. 774.
- (13) SAVAGE (1912). *Milk and the Public Health*, p. 238.
- (14) KINYOUN, J. J. (1914). *U.S. Naval Med. Bull.* VIII. 435-42; abstr. in *Expt. Sta. Rec.* 31 (1914), 574.
- (15) AYERS, S. H., COOK, L. B. and CLEMMER, P. W. (1917). *Abstr. Bact.* I. 52-53; abstr. in *Expt. Sta. Rec.* 37 (1917), 874.



## THE WILSON-WEIL-FELIX REACTION IN TYPHUS FEVER<sup>1</sup>.

By W. JAMES WILSON, M.D., D.Sc., D.P.H.

(A REPORT TO THE MEDICAL RESEARCH COUNCIL.)

*From the Public Health Laboratories, Queen's University, Belfast.*

IN the first part of this communication I have shown that in Ireland it is possible to diagnose Typhus Fever by means of a serological test, thereby confirming the results obtained in Germany, Austria, Russia, Turkey, Egypt, Palestine, Mesopotamia, France, Italy, Spain, Holland, and Peru. In the second part I have traced the development of the test and have endeavoured by a review of the literature of the subject to offer an explanation of a phenomenon which opens up new ground in the field of immunity.

### PART I.

Most authorities believe that the Widal test is of value in distinguishing between Typhoid Fever and Typhus Fever. From a wide experience of the test in many outbreaks of Typhus Fever I can state that in certain of these the Widal test is uniformly negative whilst in others it is in a considerable number of cases as markedly positive as if the patients were suffering from Enteric Fever. I had reached this conclusion in 1908 as the result of investigations made in conjunction with Dr E. H. Milligan. At the same time Patterson (1908) in Lanarkshire showed that the blood serum of Typhus Fever cases can agglutinate the Typhoid bacillus in high dilutions.

In 1909, I pointed out that in certain cases the sera of Typhus Fever not only agglutinated the Typhoid bacillus but also a late lactose-fermenting coliform bacillus isolated from the intestine of one of the patients. This coliform bacillus was subsequently proved to be identical with a bacillus isolated by T. Horiuchi from cases of Manchurian Typhus Fever and which were thought by this observer to be due to infection with his bacillus. I however contented myself with the statement: "The fact that the blood serum of typhus fever cases in Manchuria and in Ireland should have been independently discovered to have an agglutinative action on an intestinal organism is rather interesting, but whether the phenomenon should be taken as an instance of specific or of heterologous agglutination we must leave for the present undecided."

<sup>1</sup> An abstract of this paper was read before the Pathological Society of Great Britain and Ireland at its meeting in Edinburgh in July 1919.

In 1910, in a paper on the Etiology of Typhus Fever, I pointed out that:

(1) From the faeces of one case a variant form of *B. coli communis* was cultivated on which the blood serum of 17 Typhus Fever cases was found to have three to ten times the agglutinative effect of normal serum.

(2) From the urine of two cases a bacillus resembling *B. coli communis*, but having no action on lactose, was cultivated. This bacillus formed blue colonies on the Conradi-Drigalski medium and was regarded by me as an intestinal micro-organism but whether it belonged to the *B. coli* or *B. proteus* group was not determined. This bacillus was agglutinated in dilution of 1 in 50 and 1 in 100 by the serum of the cases but not by normal serum. As to the interpretation of such facts I stated: "The results obtained by Horiuchi, Patterson and ourselves definitely prove that in Typhus Fever agglutinins for the typho-coli group of micro-organisms are present in the blood serum of the patients, but the knowledge which has recently been acquired with regard to the presence of heterologous agglutinins in Cerebro-Spinal fever prevents us from drawing the unwarranted conclusion that the presence of a bacillus in the intestine and urine and the discovery of agglutinins for it in the blood indicate that such an organism is the cause of the disease in question. Though future research may show that the causative organism of Typhus Fever is in no way related to diplococci or to any variety of intestinal organism still the presence of agglutinins for these organisms in the serum probably indicates that the latter are infecting the patient. We are now beginning to learn that the body in infection has not only to deal with the specific microbe and its toxins but also with certain organisms contained in the alimentary canal which are normally saprophytic but which in the altered conditions of metabolism produced by disease become to some degree pathogenic."

From the above references it is obvious that as long ago as 1908 I established the presence of heterologous agglutinins for intestinal bacilli in the blood serum of Typhus Fever cases. The recent work on the Weil-Felix reaction is an amplification of my investigations, the basis of the test being the demonstration of heterologous agglutinins. The micro-organism employed by Weil and Felix is a proteus bacillus whereas of my cultures the intestinal were probably genuine varieties of the *B. coli* and the urinary varieties of *B. proteus*. It would appear from the wide use of "X 19," the strain of *B. proteus* most commonly employed by Weil and Felix and others, that this bacillus is the most suitable for the detection of these agglutinins.

In view of my initial work on the subject I would propose that the test should be known as the Wilson-Weil-Felix reaction.

Through the kindness of Dr Arkwright I was able to obtain a culture of "X 19" and to employ it in the investigation of the blood sera of 24 cases of Typhus Fever which occurred in Londonderry during December 1918 and in the early months of 1919. Dr Craig the Medical Officer of Health of Londonderry very kindly supplied me with specimens of blood.



The results of my observations may be summarized as follows:

(1) Of 23 cases examined the agglutinative titre of the sera against an emulsion of "X 19" was as follows: one in 1 in 40, four in 1 in 80, five in 1 in 160, three in 1 in 320, four in 1 in 640, one in 1 in 1280, four in 1 in 2560. Most of these observations were made during the second week of the disease. In one case the reaction was negative in 1 in 20 dilution on the 5th day of the disease but a week later agglutination occurred in a dilution of 1 in 160. One case examined on the 12th and again on the 14th day was negative: the death of this patient prevented further examination. As controls sera from the following gave completely negative results in a 1 in 20 dilution: 50 cases of Influenza, 12 cases of Trench Fever and 15 cases of Syphilis.

(2) The agglutinative reaction is slow when cultures killed by heat and preserved with 0.1 per cent. formalin are employed. Results can be read after 20 hours at room temperature or after 12 hours at room temperature followed by two hours at 55° C. One of the cases showed a definite "Zone of Inhibition," agglutination being absent in dilutions of 1 in 20 and 1 in 40 but present in 1 in 80, 1 in 160, etc.

(3) The examination of 10 sera convinced me that the formation of agglutinin for this proteus-like organism in the blood of typhus cases is not accompanied by the formation of immune body, as indicated by the complement fixation reaction using as antigen a fresh saline suspension of this proteus-like organism. This is confirmatory of the work of Craig and Fairley (1918) and differs from that of some Continental observers. In carrying out the test the sera were exposed to the antigen for different periods and at different temperatures.

(4) The titre of the agglutinins in the sera of 20 cases for a coliform bacillus isolated from the urine of one of them was: one in 1 in 640, five in 1 in 320, five in 1 in 160, six in 1 in 80, three in 1 in 40. The blood serum of 50 Influenza cases and of 12 Syphilitics gave feeble agglutination in a 1 in 20 dilution at 55° C. but none in 1 in 20 at room temperature. In the case of no non-typhus serum was there agglutination with a 1 in 40 or higher dilution. The agglutinins for this coliform bacillus were best demonstrated at 50° C. This bacillus was a genuine *B. coli*. It fermented lactose and was most readily agglutinable when grown on lactose agar. Other strains of *B. coli* which were isolated from Typhus cases gave no agglutination.

(5) Absorption experiments indicated that the agglutinins for *B. proteus* "X 19" and this coliform bacillus were distinct.

(6) The agglutinins in Typhus serum for *B. proteus* "X 19" are completely destroyed by heating for half an hour at 60–65° C. whereas the specific agglutinins for the same bacillus in the serum of an inoculated rabbit are not destroyed until a temperature of 75° C. is reached.

These results confirm those of everyone who has used the test. In my controls the results were always negative in a 1 in 20 dilution. I had an opportunity through Col. H. L. Cummins kindly supplying me with Schiff's

Diagnostikum—a dead suspension of “X 19”—to test the action of Trench Fever serum on the bacillus. I had early in 1917 pointed out certain points of resemblances between the two diseases but my experiments showed me that as regards the Wilson-Weil-Felix test they were quite different. Jungmann and Kuczynski (1917) had also remarked on the close relationship of these diseases and later Kuczynski stated that he had seen “X 19” agglutinated by the serum of Volhynian Fever. I may mention that I endeavoured to develop a similar test for the diagnosis of Trench Fever, using cultures obtained from the urine of the cases, and, although I found that there was an increase in their blood of agglutinins for a bacillus which in cultural and fermentative characters resembled *B. paratyphosus* B, but which differed from the latter in forming indol, still the serum in some other conditions showed the same alteration and rendered the test unreliable.

In a case in which the clinical diagnosis of Influenza and Typhus Fever was in question I was able to decide the matter by finding agglutination of *B. influenzae* in a 1 in 160 dilution and a negative Wilson-Weil-Felix in a 1 in 20 dilution.

Other observers have used as controls the sera of patients suffering from the common tropical diseases and as a rule where Typhus infection, recent or remote, could be excluded the test was negative. The chief exception is Enteric Fever where agglutination is sometimes found in dilutions of 1 in 50 and even in 1 in 100.

From a perusal of the literature it is obvious that there has been a difference in the agglutinability of the strains of *B. proteus* “X 19” which have been employed by different observers. Indeed Diehl (1918) has already called attention to this variability. Schiff (1919) states that when “X 19” has been cultivated for some time on sugar-free media, it loses to a great extent its agglutinability and this reappears on the addition of glucose. When the bacillus is grown in a medium rich in glucose it becomes spontaneously agglutinable. Csernel (1918) claims to have rendered ordinary strains of *B. proteus* agglutinable by Typhus serum by cultivating them on lactose agar together with acid-producing faecal bacteria. I observed that strains of *B. coli* which were agglutinable by Typhus serum were rendered still more agglutinable by cultivating them on lactose agar, the lactose being fermented by them. In carrying out the test care should be taken to see that the strain of *B. proteus* “X 19” used is of a suitable degree of agglutinability. In view of the possibility of this characteristic being lost or modified by continued cultivation I have experimented with emulsions prepared from dried bacterial substance. I believe that in the dried state the bacteria preserve their agglutinable characteristics unaltered and that from such dried bacterial substance suitable emulsions can be employed for carrying out the test when a case for diagnosis crops up.



## PART II.

## DISCUSSION.

From a consideration of the very extensive literature dealing with the serology of Typhus Fever and from my own observations, it would now seem to be definitely established that the blood serum in this disease is capable of agglutinating many species of micro-organisms. Gram positive *Diplococci* (Wilson) and Diphtheroid bacilli, *B. typhi exanthematici* Platz (Baehr, Olitsky, Popoff), are frequently clumped but it is especially on certain strains of intestinal micro-organisms that the agglutination effect is manifested. The agglutination of certain coliform strains and of certain non-lactose fermenters probably of the *B. proteus* group occurring in the urine of patients is a phenomenon, as shown by me in 1908, characteristic of Typhus serum. On an average the sera of about 10–20 per cent. of the cases also agglutinate *B. typhosus*, *B. paratyphosus* A or B (*vide* Wilson, Patterson, Koehler, Zlocisti, Popoff, Mühlens and D. Stajanoff, Napier, Fairley, Werner and Leoneanu, Blanco and Tapia, Fikai, and Kramer). Nicolle in Tunis frequently found Typhus serum capable of agglutinating *Micrococcus melitensis*.

The recognition of heterologous agglutinins in Typhus serum for intestinal bacilli isolated from the intestine and urine of the cases was first made by the writer. Horiuchi (1908) a little earlier during the Russo-Japanese War had discovered a coliform bacillus in the urine of cases of Manchurian Fever and found that this bacillus was agglutinated by their serum. He named his bacillus "*Bacillus febris exanthematici Mandchurici*" and regarded it as the cause of the outbreak in question but whether the outbreak was to be regarded as true Typhus Fever Horiuchi left in *dubio*. The question of the agglutinins being heterologous in nature never entered his mind.

It was however the work of Weil and Felix (1916) and their followers which firmly established and led to the general recognition of the presence of heterologous agglutinins as diagnostic of Typhus Fever. The bacillus which was discovered by them and which has been found most admirable for the detection of these agglutinins in Typhus serum is a strain of *B. proteus* and has been named "X 19." The strain "X 2" which they first used and which was probably similar to that isolated from urine by me was found not to be so readily agglutinable as the latter strain. The distinction between a non-lactose-fermenting coliform bacillus and a *B. proteus* can only be made by a complete study of the micro-organisms. It is now recognised that the proteolytic power of *B. proteus* is variable—the majority liquefy coagulated serum and gelatin but some strains attack neither—others digest serum but do not liquefy gelatin (Schaeffer). Indol production is a variable character. All the *B. proteus* X strains produce indol and this indol producing capacity seems to be associated with the power to ferment maltose and saccharose (Schaeffer). Wenner and Rettger (1919) from a study of the genus *Proteus* propose that it should be divided into two species, *Proteus vulgaris* and *Proteus mirabilis*,

the former fermenting maltose and saccharose with the production of acid and gas, the latter not fermenting maltose and only very slowly, if at all, fermenting saccharose. Mannite and lactose are not fermented by *B. proteus*.

*B. proteus* "X 19" ferments glucose, laevulose, galactose, maltose and saccharose, liquefies gelatin and belongs to the *Proteus vulgaris* species. "In their agglutination power the members of the *Proteus* genus are heterogeneous in character so that no distinct separation into species is possible on this basis" (Wenner and Rettger). Braun and Salomon in a study of 36 *Proteus* strains of non-Typhus origin and of ten strains sent by Weil put them in three categories: (1) those possessing almost no agglutinogens common with the *Proteus* of Typhus, (2) those having agglutinogens common with all the Typhus strains, (3) the Typhus strains.

The great and only characteristic of the "X" strains and especially of "X 19" is that they are agglutinated by the serum of Typhus Fever cases. Typhus serum does not agglutinate ordinary *Proteus* bacilli, although among the latter there are strains which are agglutinated by the anti-serum of man or rabbit prepared against the "X" strains (Weil and Felix).

It is a curious fact that, although ordinary *vulgaris* strains of *B. proteus* have been isolated from Typhus cases, no agglutinins are found for these strains in the patients' sera, although the sera possess a high agglutinin content for "X" strains and all attempts in most instances to isolate the latter from patients' bodies have failed. This circumstance appears to the writer to militate against the view that in Typhus there is always specific infection with *Proteus* bacilli and that this infection is the cause of the appearance of the agglutinins in the sera. If this infection occurred it is surprising that it is for the "X" strains alone that agglutinins are found.

Another argument against the view that the agglutinins are indicative of infection is the rarity with which "X" strains have been isolated from the cases. Thus Schürer and Wolff (1919) state that Felix attempted without success on 419 occasions to cultivate *Proteus* bacilli from 250 cases of Typhus, that Zeiss in 277 blood samples found them 18 times and that Wolff out of 78 attempts was successful eight times. Schürer and Wolff (1919) in 250 blood samples from 260 typhus cases with high fever found *B. proteus* 20 times, i.e. 7.7 per cent. of the cases examined. Of these 20 only seven corresponded to "X 19" type of Weil and Felix and three to their "X 2" type.

Schürer and Wolff (*loc. cit.*) examined 450 catheter specimens of urine from 95 cases of Typhus and found *Proteus* bacilli 137 times but of these only 16 showed an agglutination with Typhus serum in higher dilutions than those of 72 strains of *Proteus* isolated from patients not suffering from Typhus Fever but from Enteric Fever, and Dysentery.

It is clear then that "X" strains are not frequently met with in Typhus Fever and they are not peculiar to Typhus but have been found in the intestine of individuals who never suffered from this disease. Georg Wolff (1919) found "X" strains three times among 116 *Proteus* strains isolated from non-



typhus patients. This is a point against the view that the "X" strains are ordinary *Proteus* strains modified by growth in the body of Typhus Fever patients.

It may be asked are these agglutinins in any way different from agglutinins developed as the result of inoculation of man or animal with "X 19"? Hamburger and Bauch (1917) believe that the agglutinins found in Typhus serum are true agglutinins but that they differ from specific agglutinins formed as a result of inoculation in that the former are completely destroyed in half an hour at a temperature of 65° C., the latter only at 75° C. This greater susceptibility to heat has been confirmed by Jacobitz (1918) and Wilson. Hamburger and Bauch found that like other agglutinins the typhus agglutinins were precipitated from the sera by ammonium sulphate and were absorbed by animal charcoal and by emulsions of the bacilli in question.

A difference between the Typhus agglutinins and specific agglutinins resulting from inoculation with "X 19" is manifested with regard to their action on heated and unheated bacilli. Dietrich (1916) showed that if emulsions of "X 19" were used which had been heated to 56° C. or which had been treated with phenol or formalin little or no agglutination occurred. Sachs confirmed this and states that the microbes become agglutinable again when the temperature is raised to 80° C. and that the bacteria now preserve their agglutinability longer than non-heated emulsions and that they are agglutinated in a higher titre although the agglutinative action is slower. Schiff found that cultures heated to 100° C. for two minutes are generally well agglutinated and that bacilli rendered inagglutinable by heating to 56° C. recover their agglutinability on being washed with normal saline solution.

Weil and Felix found "X 1" and "X 2" more agglutinogenic and much less agglutinable than "X 19" by Typhus serum. Sachs showed that the anti-sera of rabbits agglutinate all three to the same degree. When however the bacilli are heated the results differ—thus the anti-sera of "X 1" and "X 2" which agglutinate the corresponding microbes either living or heated, no longer agglutinate "X 19" when the latter has been heated: at the same time "X 1" and "X 2" become after heating inagglutinable to the serum of "X 19."

As regards the presence and production of agglutinins for "X 2" and "X 19" Felix had an interesting experience. From the cadavers of 18 Turks who had died from Typhus Fever, Felix failed to isolate any *Proteus* strains in ten, in five he found ordinary *Proteus* strains and in only three "X" strains and of these two belonged to the "X 2" and one to the "X 19" group. Now the curious fact emerged that the blood serum of the two cases which harboured "X 2" failed to agglutinate this bacillus, but agglutinated "X 19" whilst the subject from whom "X 19" was derived agglutinated both strains.

In infections with most micro-organisms the presence of an immune body can be demonstrated by means of the complement fixation test. If there is a true infection in Typhus with *Proteus* bacilli one would expect to find

complement fixed when "X 19" is used as antigen. It is unfortunate that the results obtained by different observers are conflicting and that further investigations will be required to settle the point. I failed to find any fixation and in this I am in agreement with Fairley, Otto, Dietrich and Orticoni.

Fairley's investigations were most exhaustive and would seem to be conclusive as regards the strain of "X 19" he was using as antigen. His results showed: (1) no increased tendency for fixation of complement by a pooled Typhus serum in the presence of *B. proteus* antigen over that quantity fixed by a pooled negative serum under similar conditions; (2) 55 out of 58 cases of definite Typhus Fever yielded negative complement-fixation reactions; (3) using an identical technique, monkeys and man, after subcutaneous inoculations, invariably yielded positive complement-fixation reactions. Fairley states his conclusions as follows: "In consequence of these findings and in contra-distinction to the generally accepted view, I hold that the only satisfactory explanation of the Weil-Felix-agglutination reaction is to regard the phenomenon as due to a secondary heterologous agglutinin (Neben-agglutinin)."

On the other hand Friedberger (1917), von Gutfeld (1919), Werner (1918), Reichenstein (1917) and Papamarku (1917) found complement-fixation occurred with "X 19" in the majority of cases. Papamarku states that an antigen in which the bacilli were killed by phenol gave better results than where heat was employed.

As regards other effects of Typhus serum on *Proteus* "X 19" it may be mentioned that Lisbonne and Carrère (1919) found that it caused a precipitate with the filtrate of cultures of *Proteus* "X 19."

Weltmann (1917) states that when fresh Typhus serum is added to five times its volume of distilled water a turbidity occurs and that this is absent with normal serum and with Typhus serum which has been heated for ten minutes at 60° C.

Vaglio (1919) states that when *Proteus* "X 19" is grown in broth containing 1 per cent. of Typhus serum the bacilli are deposited as clumps at the bottom of the tube after three hours incubation at 37° C.

As to the explanation of the presence of agglutinins for *Proteus* "X 19" in Typhus serum hardly anyone now considers that this micro-organism is the cause of Typhus. Apart from other arguments the negative results of the human inoculation experiments of Kuczynski and Fairley are conclusive.

The great majority of those who have studied the subject incline to the view that the agglutinins are due to a constant secondary infection with *Proteus* "X 19." This may be true but we have seen in the preceding discussion some points against this hypothesis. I have found (and this is a matter that has recently been confirmed by Schaeffer) that agglutination of certain strains of *B. coli* is almost as constant and as specific an effect of Typhus serum as the agglutination of *Proteus* "X 19." I have also found that absorption by "X 19" leaves agglutinins for *B. typhosus* and for agglutinable *B. coli*



strains unaffected. Absorption with *B. typhosus* and with the *B. coli* strains that I have studied also leaves the agglutinins for *Proteus* "X 19" undiminished in amount. If these agglutinins for intestinal micro-organisms are due to a subinfection (and it is difficult to understand how otherwise they could arise) then the infecting micro-organism must be a species that is constantly present in the bodies of the patients. It would appear that "X" strains are rarely met with and that it is unusual to get agglutinins for the *Proteus vulgaris* strains which are frequently found in the human intestine. It appears to the writer probable that the Typhus Fever virus renders the intestine more permeable to intestinal micro-organisms, and that certain strains of *B. coli* or allied organisms infect the patient and lead to the formation of specific agglutinins for themselves and of paraggglutinins for other intestinal micro-organisms, e.g. *Proteus* "X 19," "X 2," coliform bacilli, *B. coli*, *B. typhosus*, *B. paratyphosus* A and B, *Micrococcus melitensis*, etc. It is of some bearing in this connection that occasionally in Enteric Fever there is agglutination of *Proteus* "X 19" in 1 in 100 dilution. When this micro-organism is discovered and isolated it will probably be found that it produces a specific agglutinin and immune body for itself and paraggglutinins for a great variety of other bacteria and that absorption of the serum with this culture will remove not only the specific agglutinins but also the paraggglutinins.

It has been proved by Möllers and Wolff (1919) and by Ribeyro (1919) that inoculation of guinea-pigs with Typhus virus is not followed by the development of agglutinins for "X 19." Doerr and Pick (1919) state that when rabbits were inoculated with Typhus virus, they presented no signs of infection but that the Typhus virus became located in their brains and their blood serum agglutinated *Proteus* "X 19" and *B. typhosus* in dilutions up to 1 in 60 and 1 in 40 respectively.

Some light is thrown on the Wilson-Weil-Felix reaction in Typhus Fever by the study of the formation of group and heterologous agglutinins in other diseases. Kligler (1918) reported the cross-agglutination of *B. coli communis* and *B. dysenteriae* Shiga. The end point of the titre of the serum of inoculated rabbits was in each case 1 in 4000. The property of reciprocal agglutination was limited to the two strains described. Other cultures of *B. coli* and *B. dysenteriae* Shiga did not exhibit it. Absorption experiments made with each culture upon each kind of immune serum indicated that two distinct agglutinins were yielded in about equal amount in the process of immunization of rabbits with the respective cultures. The two agglutinins were specific ones, each for its own culture, and accessory (paraggglutinin) each for the other culture. The absorption of the accessory agglutinin left the specific agglutinin quantitatively unaffected, but absorption of the specific agglutinin by its own culture completely removed the paraggglutinin.

Park and Williams (1910) observed that the serum of a horse immunized with *B. dysenteriae* Flexner agglutinated *B. coli* in the same end dilutions (1:10,000) as the dysenteric bacillus. Conversely a goat immunized with

*B. coli* yielded a serum of a titre of 1 : 5000 for the *B. coli* and 1 : 3000 for *B. dysenteriae* Flexner.

Of great interest is a paper by Frost (1910) on *Pseudomonas protea* which he isolated from water and found to be agglutinated by the serum of Typhoid Fever patients. This organism which is closely allied to *Proteus* was agglutinated by the serum of Typhoid Fever cases in a larger percentage of cases than was *B. typhosus*, the difference being especially marked in the early stages of the disease. The serum of cases of Typhoid Fever often agglutinated *Ps. protea* in higher dilutions than it agglutinated *B. typhosus*. The serum of four cases of Paratyphoid Fever also agglutinated the micro-organism. Specific Typhoid immune-serum from animals in the early states of immunization, agglutinated *B. typhosus* (1 in 2000) but later as the agglutinating strength for *B. typhosus* increased, the titre for *Ps. protea* failed to increase proportionately. The results of absorption experiments indicated that the agglutination of *Ps. protea* by specific Typhoid agglutinating serum is effected by combination with a portion of the specific Typhoid agglutinin; that it was therefore a "group" agglutinin. Animals injected with culture of *Ps. protea* developed agglutinins for this organism but none for *B. typhosus* or other organisms of the Colontyphoid group.

These observations of Frost would tend to support the view put forward in this paper that the agglutinins for *Proteus* "X 19"—an organism closely allied to the *Ps. protea*—may prove to be group agglutinins of some organism of the Colon group which is a constant inhabitant of man's intestine. It is noteworthy in Frost's experiments that inoculation with his *Ps. protea* produced no group agglutinins for *B. typhosus* but that inoculation with the latter produced group agglutinins for the *Ps. protea*.

In an endeavour to fix in its true setting the Wilson-Weil-Felix reaction in the field of Immunity, I believe help may be derived from the contents of our three papers in the *Journal of Hygiene* (Symmers and Wilson, 1908; Wilson, 1909 and 1910). The following quotations from the second paper appear to me to bear on the problem under discussion: "In round numbers 90 % of the cases of Cerebro-spinal fever agglutinated the *B. aquatilis alkaligenes* in a dilution of 1 in 50. In most cases agglutination still occurred with a 1 in 100 and higher dilutions. In 16 cases the blood serum gave marked 'clumping' within an hour to dilutions of 1 in 1000 and further we found that one of the 16 agglutinated in 1 in 1400, two in 1 in 1500, four in 1 in 1600 and four in 1 in 2000 dilutions respectively. We have shown that it is possible to remove the agglutinins from the serum by saturation with the *B. aquatilis alkaligenes*, whilst saturation with the *Meningococcus*, *B. typhosus*, *B. coli communis*, or *B. faecalis alkaligenes* (Král) leaves them intact."

"Of 31 specimens of the serum of normal adults examined three, i.e. 9.6 per cent. gave a positive reaction in a 1 in 50 and all a negative reaction in a 1 in 100 dilution of the serum." The *B. aquatilis alkaligenes* was a non-pathogenic micro-organism which had been isolated from tap water. This curious instance



of heterologous agglutination by means of which we were enabled to diagnose cases of Cerebro-spinal Fever is worthy of note.

At that time (1908) I succeeded in only one case in isolating a similar bacillus from the blood of the patients and my examinations of the Cerebro-spinal fluid, urine and blood were negative as regards the *B. aquatilis alkaligenes*. However, in 1915, in an outbreak of Cerebro-spinal Fever, I frequently found associated with the *Meningococcus* in the spinal fluid a bacillus which was evidently of intestinal origin and which fermented no sugar but usually liquefied gelatin. This bacillus although allied to the *B. aquatilis alkaligenes* was not agglutinated by the serum of the cases in nearly so high a titre as the latter. It in fact bore much the relationship to the *B. aquatilis alkaligenes* that *Proteus* "X 1," "X 2" and ordinary *Proteus vulgaris* strains bear to "X 19."

"Paltauf (1904) says the results of Posselt and Sagasser (1903) as well as those of Hetsch and Lentz go to show that in the immune serum of animals as well as in that of sick men and women, heterologous agglutinins exist which have no binding groups for the infecting bacteria but are as specific as regards absorption as those developed in a mixed infection. They must therefore be distinguished from partial agglutinins or 'mitagglutinins.' They can be designated as 'heterologous nebenagglutinins' or more briefly as 'neben-agglutinins.' For their formation the views held regarding partial agglutinins do not apply." "The explanation of the fact that the blood serum of Europeans contains specifically absorbable agglutinins for the *V. cholerae* and *B. pestis* may be that these agglutinins are partial agglutinins or 'nebenagglutinins' caused by the action of unknown saprophytic intestinal organisms."

I would suggest that a similar explanation may account for the agglutinins present in Typhus serum for *Proteus* "X 19," etc. With regard to the agglutinins in Cerebro-spinal Fever for *B. aquatilis alkaligenes* and *B. typhosus* I concluded that "these secondary agglutinins do not indicate a mixed infection but are of the nature of heterologous 'nebenagglutinine.'" I proved that they were not partial agglutinins due to infection with the *Meningococcus* but I now think it not improbable that they were of the nature of partial agglutinins for some unknown intestinal micro-organism. I indeed stated that "the explanation of the production of heterologous agglutinins may be that infection with certain germs leads to an alteration of the bacterial flora of the intestine. As the result of this secondary auto-infection, along with the agglutinins for the primary infecting organism, agglutinins are formed for the intestinal micro-organisms also." Experiments conducted by Elser and Huntoon tend to confirm the statements made above and to offer a similar explanation as is made clear by the following extract from their paper: "In rabbits we have shown that injections of the *Meningococcus* render the mucosa of the intestinal tract more permeable for the Typhoid bacillus (or its products) administered with the food. Animals thus treated developed agglutinins for the organisms administered by mouth, while no augmentation of the agglutinins was

observed in the controls which were fed with Typhoid bacilli but received no injections. These experiments suggest an explanation for the frequent occurrence of mixed infections in animals receiving experimental inoculations of the meningococcus, and also offer an explanation for the appearance of heterologous agglutinins in the sera of such animals and in the sera of individuals suffering from epidemic cerebro-spinal meningitis."

It is pertinent to note that with regard to the heterologous agglutinins occurring in Cerebro-spinal Fever I had shown that (1) they are bound up with the globulin component of the serum, the greater part belonging to the "pseudo-globulin" fraction; (2) by heating the serum to 60° C. their destruction is complete. Similar results have been obtained in the case of the agglutinins for "X 19" in Typhus serum.

Before concluding I should mention that Epstein regards the different reactions given by Typhus serum as due to changes in the physico-chemical state of the serum. He has shown that the albumin-globulin ratio in Typhus varies from 0.46 to 0.8 whereas in other pathological states it exceeds unity. However it is difficult to conceive how apart from subinfection with intestinal bacilli this change should lead to the production of absorbable agglutinins for these micro-organisms. No doubt the action of agglutinins is best explained in terms of physical chemistry but an explanation of their production apart from infection with some allied micro-organism is difficult. The agglutinins for *B. aquatilis alkaligenes* I showed were heterologous in the sense that they were not partial agglutinins, group agglutinins, or agglutinins due to infection by the *Meningococcus* acting alone. The agglutinins in Typhus serum are also heterologous as regards their production by the virus of Typhus but in both instances I would suggest that they are due to subinfection from the intestine. The infecting micro-organisms however have, so far, not been definitely determined and are unlikely to be *B. aquatilis alkaligenes* or *Proteus* "X 19" but some constant inhabitants of the intestine which under the stimulus of the disease produce specific agglutinins for themselves and group agglutinins for numerous other micro-organisms especially for *Proteus* "X 19," *Proteus* "X 2," and various members of the Colon-typhoid group.

#### SUMMARY.

1. The Wilson-Weil-Felix agglutination reaction with cultures of *Proteus* "X 19" was Positive in dilutions of the serum ranging from 1 in 40 to 1 in 2560 in 22 out of 23 cases of Typhus Fever occurring in Ireland. In one case the reaction was still negative at the time of the patient's death on the 14th day of the disease. As controls, sera from the following gave completely negative results in a 1 in 20 dilution: 50 cases of Influenza, 12 cases of Trench Fever and 15 cases of Syphilis.

2. The agglutinins in Typhus serum for *B. proteus* "X 19" are completely destroyed by heating at 65° C. for half an hour.



3. Ten sera were tested for presence of immune body by means of the Complement Fixation test. The results were negative.

4. A coliform bacillus isolated from the urine of one case was agglutinated by the sera of twenty cases—the only ones examined—in dilutions varying from 1 in 40 to 1 in 640.

Sixty-two controls gave negative results with a 1 in 40 or higher dilution of their sera.

5. Absorption experiments indicated that the agglutinins for *B. proteus* "X 19" and this coliform bacillus were distinct.

6. The literature dealing with the heterologous agglutinins met with in the serum of Typhus Fever has been consulted and the various hypotheses that have been put forward to account for their presence have been examined. It is suggested that under the stimulus of infection with the Typhus Fever virus, some bacterium which is a normal inhabitant of the human intestine produces agglutinins for itself and group agglutinins for numerous other micro-organisms especially for *Proteus* "X 19," *Proteus* "X 2," and various members of the Colon-typhoid group.

#### REFERENCES.

- BAEHR, G. (vii. 1917). Agglutination in Typhus Fever. *Journ. Infect. Diseases*, xxi. 211.
- BALLNER, F. and FINGER, A. (19. vii. 1917). Über die Weil-Felix'sche Proteus Reaktion mit dem Harne Fleckfieberkranker. *Wiener klin. Wochenschr.* 966.
- BLANCO and TAPIA (30. ix. 1919). Serodiagnostico del tifus exantemico. Nuestra experiencia sobre la reaccion de Weil-Felix. *Bolet. del Inst. nac. de hig. de Alfonso XIII*, xv. 135.
- BRAUN, H. and SALOMON, R. (17. i. 1918). Ein Beitrag zum Wesen der Weil-Felixschen Reaktion auf Fleckfieber. *Deutsche med. Wochenschr.* 59.
- BRAUN, H. (8. vii. 1918). Das Wesen der Weil-Felixschen Reaktion auf Fleckfieber. *Berlin. klin. Woch.* 637.
- COMPTON, A. (15. xi. 1919). Studies on the Weil-Felix Serological Test. *Lancet*, 866.
- CRAIG, C. M. and FAIRLEY, N. H. (21. ix. 1918). Typhus Fever: Observations on a Serological Test. Weil-Felix Reaction. *Ibid.* 385.
- CSERNEL, E. (1918). Ueber Proteus Agglutination bei Fleckfieber. *Wien. klin. Wochenschr.* xxxi. 1036.
- DIEHL (1918). Beitrag zur Fleckfieberdiagnostik. *Med. Klinik*, xiv. 908.
- DIETRICH (21. xii. 1916). Beiträge zur Weil-Felixschen Reaktion beim Fleckfieber. *Deutsche med. Wochenschr.* 1570.
- DOERR, R. and PICK, R. (1919). Das Verhalten des Fleckfiebertivirus im Organismus des Kaninchens. *Zeitschr. f. Hyg. u. Infektionskr.* lxxxix. 243.
- EPSTEIN, E. (1918). Zur Frage der Spezifität der X Stämme und der Weil-Felixschen Agglutination bei Fleckfieber. *Ibid.* xxxi. 989.
- (1919). Zur Theorie der Serologie des Fleckfieberblutes und zur Frage der Spezifität und ätiologischen Bedeutung der X Stämme. *Centralbl. f. Bakt. Abt. I.* lxxxiii. 255.
- and MORAWETZ, G. (29. iii. 1917). Zur Serodiagnostik des Fleckfiebers. *Wien. klin. Wochenschr.* 399.
- FAIRLEY, N. H. (viii. 1919). The Laboratory Diagnosis of Typhus Fever. *Journ. of Hygiene*, xviii. 203.
- FELIX, A. (25. ix. 1917). Über die Züchtung der spezifischen Proteus-X-Stämme bei Fleckfieber. *Münch. med. Wochenschr.* 1259.

- FELIX, A. (28. xii. 1917). Serologische Untersuchungen an Fleckfieberkranken aus der Asiatischen Türkei. *Zeitschr. f. Immunitätsf.* I. Orig. xxvi. 602.
- FICAL, G. (31. iii. 1919). Osservazioni sul tifo esantematico e sulla reazione di Weil-Felix. *Ann. d' Igiene*, xxix. 145.
- FRIEDBERGER, E. (18. and 25 x., 1. xi. 1917). Ueber Immunitätsreaktionen mit dem Bazillus Weil-Felix und über seine ätiologische Bedeutung für das Fleckfieber. *Deutsche med. Wochenschr.* 1314, 1353.
- FROST, W. H. (vi. 1910). An organism (*Pseudomonas protea*) isolated from water, agglutinated by the Serum of Typhoid Fever patients. *Hygiene Laboratory, Bulletin* No. 66. Public Health and Marine-Hospital Service of U.S.A., Washington.
- VON GUTFELD, F. (1919). Die Serologie des Fleckfiebers. *Med. Klin.* xv. 691.
- HAMBURGER and BAUCH (6. ix. 1917). Untersuchungen über die Weil-Felixsche Reaktion. *Deutsche med. Wochenschr.* 1130.
- HILGERMANN, R. and ARNOLDI, W. (20. xii. 1917). Behandlung und Schutzimpfung bei Fleckfieber mittels Vakzinierung mit Proteus X 19. *Ibid.* 1582.
- HORIUCHI, T. (1908). Ueber einen neuen Bacillus als Erreger eines exanthematischen Fiebers in der Mandchurei während des japanisch-russischen Krieges, "Bacillus febris exanthematici mandchuriei." *Centralbl. f. Bakteriolog.* Orig. XLVI. 586.
- JACOBITZ, E. (1918). Untersuchungen ueber die Weil-Felixsche Reaktion mit dem Bazillus X 19. *Centralbl. f. Bakt.* Orig. LXXXI. 251.
- JUNGMANN and KUCZYNSKI (1917). Zur Aetiologie und Pathogenese des Wolhynischen Fiebers und des Fleckfiebers. *Zeitschr. f. klin. Med.* LXXXIV. 250-72.
- KLIGER, I. J. (ix. 1918). Note on Cross-Agglutination of *B. coli communis* and *B. dysenteriae* Shiga. *Journ. of Bact.* III. 441.
- KOEHLER, O. (xii. 1918). Ein Beitrag zur Serologie des Fleckfiebers. *Arch. f. Sch.- u. Tropen-Hyg.* XXII. 433.
- KRAMER, P. H. (1919). Uitkomst van de reaktie van Weil-Felix bei Vlecktyphus in de eerste twaalf dagen der Ziekte. *Nederl. Tydschr. v. Geneesk.* I. 922-3.
- KUCZYNSKI (iv. 1918). Bacterium proteus X 19 (Weil-Felix) in der Kleiderlaus. *Arch. f. Protistenk.* XXXVIII. 376.
- LISBONNE and CARRÈRE (v. 1919). Recherches Sérologiques dans un cas de typhus exanthématique. *C. R. Soc. Biologie*, LXXXII. 568.
- MÖLLERS, B. and WOLFF, G. (iii. 1919). Experimentelle Fleckfieberuntersuchungen. *Deutsche med. Wochenschr.* 349.
- MONZIOLS, A. and DUBOURG, E. (iv. 1919). Agglutination du Proteus X 19 dans le typhus exanthématique. *C. R. Soc. Biol.* LXXXII. 348.
- MÜHLENS, P. and STAJANOFF, D. (vii. 1917). Beiträge zur Weil-Felixschen Reaktion nebst Beobachtungen über die Gruber-Widal Reaktion bei Flecktyphus. *Arch. f. Sch.- u. Trop. Hyg.* XXI. 213.
- NAPIER, L. E. (xi. 1919). The Weil-Felix reaction in a mild epidemic of Typhus. *Lancet*, II. 863.
- OELTINGER, W. (1918). Zur Praxis und Theorie der Weil-Felixschen Reaktion. *Centralbl. f. Bakt.* Orig. LXXX. 304.
- OLITSKY, P. K. (vi. 1917). Immunologic studies in typhus exanthematicus. *Journ. of Immunol.* II. 363.
- ORTICONI, A. (ii. 1920). Contribution à l'étude de la séro-réaction et de la fixation du complément avec le *B. proteus* dans le typhus exanthématique. *Bull. Soc. Path. exot.* XIII. 108.
- OTTO, R. (ii. 1918). Ueber Immunitätsreaktionen mit dem Bazillus Weil-Felix und über seine ätiologische Bedeutung für das Fleckfieber. *Deutsche med. Wochenschr.* 173-174.
- OTTO, R. (vii. 1919). Die Proteus-X Bazillen und die Weil-Felixsche Reaktion beim Fleckfieber. *Ibid.* 817.



- PALTAUF, R. (1904). Agglutination in Kolle and Wassermann's *Handbuch der pathogenen Mikroorganismen*, 668.
- PAPAMARKU, P. (vii. 1917). Weitere Beiträge zur Komplementbindungsreaktion beim Fleckfieber. *Berlin. klin. Wochenschr.* 647.
- PARK, W. H. and WILLIAMS, W. (1910). *Pathogenic Microorganisms*, 166.
- PATTERSON, J. H. *Annual Report of the work done in the Bacteriological Laboratory, County of Lanark, during the year 1908.*
- POPOFF (iv. 1916). Ueber den Bazillus typhi exanthematici Plotz. *Deutsche med. Wochenschr.* 471-6.
- POPOFF, METHODI (viii. 1918). Ueber den Wert der Agglutinationreaktionen bei der Diagnosestellung der Typhus- Paratyphus- und Fleckfiebererkrankungen. *Med. Klin.* XIV. 766.
- POSSELT and SAGASSER (1903). Ueber Beeinflussung der Agglutinine durch spezif. Absorptionen. *Wien. klin. Wochenschr.* No. 24.
- RIBEYRO, R. E. (1919). El diagnostico del tifus exanthematico por la reaccion de Weil-Felix. *Cronica Medica*, xxxvi. No. 669.
- (1919). Sobre la reaccion de Weil-Felix. *Ibid.* No. 671.
- REICHENSTEIN, J. (v. 1917). Ueber Komplementbindung bei Fleckfieber mit X 19. *Deutsche med. Wochenschr.* 554.
- SACHS, H. (viii. 1917). Zur Diagnostik des Fleckfiebers. *Ibid.* 964.
- (iv. 1918). Zur Kenntnis der Weil-Felixschen Reaktion. *Ibid.* 459.
- SCHAEFFER, H. (1919). Untersuchungen über Proteusbazillen. Zugleich ein Beitrag zur Theorie der Weil-Felixschen Reaktion. *Centralbl. f. Bakt.* I. Abt. LXXXIII. 430.
- SCHIFF, F. (x. 1917). Ein haltbares Präparat zur Fleckfieber Reaktion nach Weil-Felix. *Deutsche med. Wochenschr.* 1292.
- (ii. 1919). Zur Agglutinabilität des Weil-Felixschen Bazillus. *München. med. Wochenschr.* 152.
- SCHILLING, V. (v. 1919). Das Zusammenwirken von Blutbild und Weil-Felix Reaktion bei der Laboratoriumsdiagnose des Fleckfiebers. *Ibid.* 486.
- SCHÜRER, J. and WOLFF, G. (1919). Ueber die Bedeutung der Proteus-Bazillen beim Fleckfieber. *Centralbl. f. Bakt.* I. Abt. Orig. LXXX. 517.
- SEYFARTH, C. (xi. 1918). Die Weil-Felixsche Reaktion bei Malaria und Recurrens und Mischinfektionen dieser Krankheiten mit Fleckfieber. *Med. Klin.* 1913.
- STERLING, ST. and K. (vii. 1917). Beitrag zur Weil-Felixschen Reaktion. *Wien. klin. Wochenschr.* 972.
- SYMMERS and WILSON (vi. 1908). Agglutination of Bacilli of the Alkaligenes, Colon and Typhoid groups by the Blood Serum of Cerebro-Spinal Fever. *Journ of Hygiene*, VIII. 313.
- VAGLIO, R. (vi. 1919). L' agglutinazione allo stato nascente de Proteus X 19 nella diagnosi sierologica del tifo essantematico. *Pathologica*, xi. 232.
- VALLARDI, L. (ix. 1919). Sulla reazione di Weil-Felix nel tifo essantematico. *Ibid.* 379.
- VIALATTE, COLLIGNON and BÉNARD (v. 1919). La réaction de Weil-Felix dans le typhus exanthématique. *Bull. Soc. Méd. Hôp.* 484.
- VIOLLE, H. (iii. 1920). Le Typhus exanthématique et les Réactions d'agglutination. *C. R. Soc. Biolog.* LXXXIII. 283.
- VITECK, VL. (vii. 1917). Die klinische Bedeutung der Weil-Felixschen Reaktion. *Wien. klin. Wochenschr.* 967.
- WAGNER (1917). *München. med. Wochenschr.* 792.
- WEIL, E. and FELIX, A. (i. 1916). *Wien. med. Wochenschr.* XXIX. 33.
- (iii. 1917). Untersuchungen über das Wesen der Fleckfieber-agglutination. *Wien klin. Wochenschr.* 393.

- WEIL, E. and FELIX, A. (1918). Untersuchungen ueber die Proteusstämmen und ihre Beziehungen zu den X Stämmen. *Centralbl. f. Bakt. I. Orig.* LXI. 637.
- WELTMANN, O. (iii. 1917). Weiterer Beitrag zur Serologischen Fleckfieberdiagnose. *Wien. klin. Wochenschr.* 408.
- WENNER, J. J. and RETTGER, L. F. (vii. 1919). A Systematic study of the Proteus Group of Bacteria. *Journ. of Bact.* iv. 331.
- WERNER, H. and LEONEANU, E. (v. 1918). Zur Serologie des Fleckfiebertyphus. *München. med. Wochenschr.* LXIII. 587.
- WILSON and MILLIGAN (1908). Bacteriological and Cytological investigations of the Blood in Typhus Fever Cases. *Trans. Ulster Med. Soc.* Session 1908-9, p. 62.
- WILSON, W. J. (1909). On heterologous agglutinins more particularly those present in the Blood serum of Cerebro-Spinal Fever and Typhus Fever Cases. *Journ. of Hygiene*, ix. 316.
- (1910). The Etiology of Typhus Fever. *Ibid.* x. 155.
- PURCE, R. B. and DARLING, G. (xii. 1916). Report on certain points observed with regard to Cerebro-Spinal Fever in the Belfast Military District during 1915 and up to November, 1916. *Brit. Med. Journ.*; also *Trans. Ulster Med. Soc.* 1915-16.
- (vi. 1917). Typhus Fever and the so-called Weil-Felix reaction. *Brit. Med. Journ.* 825.
- WOLFF, G. (xi. 1917). Zur Aetiologie der Weil-Felixschen Reaktion. *Deutsche med. Wochenschr.* 1507.
- (vi. 1919). Fehlerquellen der Weil-Felix Reaktion. *Münch. med. Wochenschr.* 507.
- (v. 1919). Zur Kenntnis der Verbreitung agglutinabler Proteusstämmen des Typhus X 19. (Weil and Felix.) *Berl. klin. Wochenschr.* 483.
- ZLOCISTI, T. (viii. 1918). Die Beziehungen des Widal-Gruber zum Fleckfieber und zur Weil-Felix-Reaktion. *Beihefte z. Arch. f. Sch.- u. Tropen-Hyg.* XXII. 88.



## THE SECOND INTERNATIONAL CONGRESS OF COMPARATIVE PATHOLOGY

TO BE HELD IN ROME IN APRIL, 1921.

The following letter having reached me, after my visit to Rome in April, 1920, I take this means of making its contents known to those desiring to participate in the work of the Congress.

The Italian Committee of Organization has adopted the French language for all foreign correspondence and printed matter concerning the Congress.

Rome, 1<sup>er</sup> juin 1920.

*Monsieur et très honoré Confrère,*

Les Congressistes réunis à Paris en Octobre 1912 pour le I<sup>er</sup> Congrès International de Pathologie Comparée, ont désigné la ville de Rome pour siège du II<sup>ème</sup> Congrès.

À la suite de la guerre ce Congrès, qui devait se tenir en 1914, a dû être renvoyé; le Comité d'organisation a maintenant établi que le II<sup>ème</sup> Congrès International aie lieu dans le prochain printemps du 1921.

Puisque vous avez contribué avec autant d'autorité que d'activité à la bonne réussite du I<sup>er</sup> Congrès International de Pathologie Comparée, il est tout naturel que nous osions nous adresser à vous, et vous prier de prendre l'initiative pour organiser dans votre pays un Comité National. Nous espérons que cette tâche ne vous donnera pas trop de peine, et que vous pourrez faire connaître au Secrétariat Général les noms des savants qui en feront part, ainsi que ceux de vos compatriotes à qui vous croyez que nous puissions envoyer un bulletin d'adhésion.

Le Congrès aura les mêmes buts qui ont été fixés pour le I<sup>er</sup> Congrès international de Paris: le règlement, qui va être imprimé et qui vous sera tout prochainement adressé, suivra à-peu-près les lignes tracées par le règlement du I<sup>er</sup> Congrès.

Nous vous adressons dès maintenant une ébauche du programme, où sont indiquées les principales questions qui pourront être objet de rapports et de communications.

Tout en vous priant de nous indiquer si quelques savants de vos compatriotes veulent bien se charger de rédiger des rapports sur les questions qui sont dans le programme, nous vous serions obligés si vous vouliez bien nous proposer d'autres thèmes d'intérêt général, ainsi que les noms des relateurs.

Lorsque vous aurez l'obligeance de nous faire part de la constitution de votre Comité National, veuillez aussi nous faire connaître s'il est préférable que toute communication soit adressée personnellement aux différents membres du Comité, ou bien toujours par la voie de la Présidence ou du Secrétariat.

Nous nous reservons de vous communiquer prochainement, avec le règlement, la date précise du Congrès, les réductions qui pourront être accordées par les chemins de fer et par les compagnies de navigation, etc.; mais dès maintenant nous vous prévenons qu'il sera nécessaire que les titres des différents rapports et des communications (sur les thèmes des rapports, ou sur d'autres sujets se rattachant à la pathologie comparée) ainsi qu'un abrégé (25 lignes de 46-54 lettres chacune) puissent par-

venir au Secrétariat Général pas plus tard que le 15 décembre 1920; à fin que l'on aie le temps de rédiger et d'imprimer le programme définitif, qui sera envoyé à tous les Congressistes.

Le droit d'admission a été fixé à 40 liras.

Toute correspondance relative au Congrès doit être adressée à M. le Prof. Mario Levi Della Vida, Secrétaire Général du Comité d'Organisation, 58 Via Palermo, Rome.

Nous vous remercions d'avance, Monsieur et très honoré Confrère, de la peine que vous voudrez bien prendre à fin de contribuer à la bonne réussite du II<sup>ème</sup> Congrès International de Pathologie Comparée; et nous vous prions d'agréer l'assurance de notre parfaite considération.

*Le Secrétaire Général*  
MARIO LEVI DELLA VIDA.

*Le Président*  
E. PERRONCITO.

### (PROVISIONAL PROGRAMME, JUNE, 1920.)

(a) Influenza de l'homme et des animaux.—(b) Fièvre aphteuse; nouvelles recherches.—(c) Cancer et sarcome. Les données actuelles sur la question du cancer.—(d) Rage et vaccination Pasteurienne; résultats.—(e) Peste bovine ou des ruminants; nouvelles recherches.—(f) Peste des poules et les dernières recherches sur les maladies des poules.—(g) Peste des abeilles.—(h) Cycle évolutif du *Dibothriocephalus latus* de l'homme et des animaux.—(i) Flusserie des vers-à-soie.—(j) Cycle évolutif des ascarides.—(k) Cycle évolutif des ankylostomes.—(l) Les piroplasmoses.—(m) Questions relatives aux acars, et la gale de l'homme et des animaux.—(n) La régénération des nerfs dans la pathologie expérimentale, dans les maladies nerveuses et dans les lésions de guerre.—(o) Symbiose et parasitisme chez les végétaux.—(p) Ténacité de vie des parasites animaux et végétaux.—(q) *Diaspis pentagona* et *Prospartella Berlesei*, et insecticides.—(r) Questions relatives au *Phylloxera*.

In a further letter from the Secretary General, dated 30 June, 1920, I am requested to send him the names of those persons throughout the British Empire who may desire to aid in the work of the Congress. Therefore, pending the formation of our Committee, communications may be addressed to me by those who are interested.

The Secretary General is prepared to receive titles and abstracts of papers dealing with Comparative Pathology of Man, Animals and Plants, provided that the communications do not reach him later than 15 Dec. 1920. Those desiring to attend the Congress are requested to communicate either with the Secretary General or with me.

GEO. H. F. NUTTALL.

Address:

PROF. GEORGE H. F. NUTTALL, Sc.D., F.R.S.,  
LONGFIELD, MADINGLEY ROAD,  
CAMBRIDGE,  
ENGLAND.



THE BEHAVIOUR OF BACTERIA IN FLUID CULTURES  
AS INDICATED BY DAILY ESTIMATES OF THE  
NUMBERS OF LIVING ORGANISMS.

BY G. S. GRAHAM-SMITH, M.D., F.R.S.,  
*University Lecturer in Hygiene, Cambridge.*

(With 29 Charts and 1 Text-fig.)

CONTENTS.		PAGE
<i>Introduction.</i>	The work of previous investigators . . . . .	134
	The scope of the experiments . . . . .	136
	Sources of error in these experiments . . . . .	138
	Method of describing the experiments . . . . .	139
Section		
I.	Growth in neutral meat extract medium . . . . .	139
II.	The influence of the previous rate of transplantation of the culture used for inoculation . . . . .	141
III.	The relation of growth to the quantity of food substance present . .	142
IV.	The influence of the numbers inoculated . . . . .	144
V.	The influence of small differences in numbers in the initial inoculation	145
VI.	The influence of occasional additions of small quantities of food substance . . . . .	146
VII.	The influence of regular additions of small quantities of food substance . . . . .	151
VIII.	The effects of diluting cultures with distilled water at various periods of growth . . . . .	152
IX.	The influence of the incubation temperature . . . . .	153
X.	The influence of the growth of one species on the growth of others subsequently inoculated into the medium . . . . .	158
XI.	The influence of varying the reaction of the medium . . . . .	164
XII.	The influence of the addition of small quantities of <i>N</i> /10 hydrochloric acid to the medium . . . . .	167
XIII.	The effects of cultivating organisms for prolonged periods on media of different reactions . . . . .	169
XIV.	The influence on growth of the addition of gelatin or agar to meat extract . . . . .	174
XV.	The effects of adding various quantities of different acids to neutral meat-extract-agar before inoculation . . . . .	177
XVI.	The influence of the addition of glucose . . . . .	179
XVII.	The effects of adding living organisms of the same species to growing cultures . . . . .	185
XVIII.	The distribution of <i>S. aureus</i> in meat extract cultures . . . . .	189
XIX.	Meat extract and pancreas extract compared as media . . . . .	190
XX.	Further experiments with media made from ox pancreas . . . . .	195
XXI.	The effects of accidental contamination . . . . .	197
	The bearing of these experiments on some of the phenomena observed in infective diseases . . . . .	198
	Summary . . . . .	200
	References . . . . .	204

## INTRODUCTION.

## THE WORK OF PREVIOUS INVESTIGATORS.

BUCHNER, LONGARD and RIEDLIN (1887) working with *V. cholerae* were the first to measure the rate of bacterial growth with any degree of accuracy. They plated cultures at the beginning of each experiment and after two and five hours' incubation and calculated the generation time, making, however, no allowance for "lag." Subsequent observers noticed that under the usual conditions of such experiments there is an initial period of no, or slow, growth—the lag-phase. Müller (1895) was the first to demonstrate the lag-phase; Hehewerth (1901) noted that it varied with the species and age of the culture employed and Rahn (1906) studied it in connection with *B. fluorescens*. Barber (1908), however, who worked with a single bacillus and studied the actual rate of division, observed that the period of lag could be abolished, if the organisms used for the inoculations were derived from rapidly growing cultures. Later observers have confirmed this observation. Lane-Claypon (1909) working with *B. coli*, *B. typhosus* and *B. enteritidis* made observations on the rate of growth of these organisms in broth cultures at temperatures ranging between 20° C. and 42° C. Only in a very few instances were the experiments continued beyond 30 hours' incubation. "The observations show that there are four phases in the bacterial life of a culture: (1) an initial period of slow or no growth; (2) a period of regular growth, the rapidity varying slightly at the same temperature, but differing widely for different temperatures; (3) a period when the numbers remain more or less stationary; (4) a period when the numbers of living bacteria are diminishing."

Period (1) varies between 1–6 hours, according to the temperature. Period (2): "For a given volume of fluid the time during which the bacteria continue to divide at a maximum rate depended upon the insemination and the temperature, being shorter if the inoculations were heavy and at the higher temperatures." Period (3): "After the culture has ceased growing logarithmically the rate of growth slackens gradually, but is still fairly active until the number of bacteria reaches several millions per drop (0.02 c.c.), that is several hundred millions per c.c. At this stage the number of living bacteria appears to remain fairly constant for some time after which it begins to decrease slowly." "There appears therefore to be a maximum number of bacteria which a unit volume of the medium is capable of supporting: this means that for the particular organism the conditions are no longer favourable for increased growth. This may be due to the using up of some constituents of the broth or it may be due to some inhibitory substance produced by the organisms themselves in the process of metabolism. I have not carried out any investigations on these points."

The nature of "lag" has been studied by a number of observers. Coplans (1910), Penfold (1914) and Chesney (1916) have shown that the period of lag differs on different media and under different conditions. Penfold has



enumerated some nine different hypotheses as to the cause of bacterial lag, all of which he regards as inadequate. He makes the suggestion that "the incubation period of infectious disease may partly depend for its existence on bacterial lag." Ledingham and Penfold (1914) published a mathematical analysis of the lag-phase of bacterial growth, and state that the logarithmic or second phase is succeeded by the third phase during which the generation time gradually lengthens till it finally becomes infinite and no further growth occurs. Subsequently the mathematical analysis of Ledingham and Penfold was elaborated by Sclator (1916, 1917).

McKendrick and Pai (1911) worked with *B. coli* and kept their cultures at a uniform temperature throughout their experiments. By using for inoculation organisms from cultures 1-3 hours old they eliminated the latent (lag) period. They state that "if there be an unlimited supply of nutriment, an organism reproduces itself by compound interest: in a geometrical progression, i.e. 1, 2, 4, 8, etc." "In test tube experiments, however, this simple state of affairs is complicated by the fact that the supply of nutriment is limited, and consequently as time goes on, the rate of multiplication falls off." "Every living organism employs the nutriment which it has absorbed for two objects; first, the maintenance of the individual; and, second, its reproduction. As, however, in the case of those micro-organisms with which we shall deal, the rate of multiplication is very fast, we may, for all practical purposes, consider that the amount of foodstuff utilised for their upkeep is negligible, and assume that the whole of it is employed in reproduction. If we accept this simplifying assumption we may say that organisms in a test tube multiply, by a simple conversion of the available foodstuff into other organisms, and that the rate of multiplication is proportional to the concentration of the foodstuff."

Buchanan (1918) made no experiments, but has published recently a study of the results secured by various authors, and states that "seven relatively distinct periods" of growth may be differentiated: (1) initial stationary phase; (2) lag-phase or positive growth acceleration phase; (3) logarithmic growth phase; (4) phase of negative growth acceleration; (5) maximum stationary phase; (6) phase of accelerated death; (7) logarithmic death phase. His first two phases seem to have been included in the lag-phase of previous workers.

Penfold and Norris (1912) made observations on the relation of concentration of peptone to the generation time, and Salter (1919) determined the rate of growth of *B. coli* for some hours and studied the effects of various dyes. The latter author observes that "a given factor may influence the rate of growth in one phase and not in another. It may cause a lengthening of the lag phase and have no influence on the logarithmic phase or may even stimulate growth during the latter phase."

All these workers have concerned themselves mainly with the earlier phases of growth and very few experiments have been published illustrating

the phases after 24-30 hours' incubation. In all cases media containing peptone were employed.

#### THE SCOPE OF THE EXPERIMENTS.

The experiments described in this paper were undertaken with the purpose of attempting to estimate the numbers of living organisms, able to multiply and form colonies after subcultivation, present at different times in fluid media during prolonged cultivation under various conditions. Since the events occurring in the earlier stages of growth under certain experimental conditions have been so carefully and so fully dealt with by previous observers, and since the events occurring in the later stages have received little attention, it was considered unnecessary to make many observations on cultures which had been incubated for less than 24 hours.

*The bacteria employed.* The organism chiefly employed was a strain<sup>1</sup> of *Staphylococcus aureus* obtained from an abscess. In some of the experiments a strain of *B. coli* and in others a strain of *B. pyocyaneus* were used.

*Media used.* The medium used in most of the experiments was meat extract made from fresh bullock's heart muscle. After the removal of the fat and vessels the meat was passed through a mincing machine and weighed. To every 100 grammes 250 c.c. of distilled water were added, and the fluid gently boiled for 90 minutes. After filtration through filter paper the clear yellow medium was sterilised for 20 minutes on three successive days in a steam steriliser. Neither peptone nor salt were added. In some of the experiments small quantities of *N/10* soda, *N/10* hydrochloric acid, agar or gelatin were added.

#### *Method of estimating the number of living<sup>2</sup> organisms present.*

In most experiments 5 c.c. of diluted meat extract (1 c.c. of meat extract to which 4 c.c. of sterile distilled water had been added) were measured by means of a sterile pipette into a sterile wide test-tube. This was inoculated with a drop of a freshly prepared emulsion in sterile distilled water of the organism grown for 24 hours at 37° C. on meat extract agar<sup>3</sup>. After violently shaking the culture to distribute the organisms (see Section XVIII) and flaming<sup>4</sup> the mouth of the tube a standard loopful of the fluid was removed and transferred to a tube of meat extract agar, which had been melted and cooled to 45° C.

<sup>1</sup> It was noticed that different strains show not only varying powers of multiplication in the same medium, but remain at a high level for different periods.

<sup>2</sup> If living organisms incapable of forming visible colonies in agar on subculture were present their numbers would not be ascertained by the method employed.

<sup>3</sup> To meat extract prepared in the manner described and made neutral to neutral red by the addition of *N/10* soda 2 per cent. of washed agar is added. After melting the agar in the autoclave the medium is filtered through cotton-wool and sterilised. No peptone, salt or egg-white are added.

<sup>4</sup> The mouths of all the tubes used were heated in the flame and the other usual precautions against accidental contamination observed.



The loopful of fluid was distributed in the agar partly by thoroughly stirring the agar with the loop and partly by rotating the tube between the hands. The contents of the tube were poured into a Petri dish, and the culture incubated at 37° C. for 24 hours or longer. The colonies growing on the medium were counted with the aid of a dissecting microscope, the Petri dish being placed on a glass plate, ruled with a diamond in the manner shown in Fig. 1 and supported on a large stage fitted with a substage mirror. To facilitate counting a recording machine operated by the finger was made use of. Each colony observed was recorded by pressing the finger and the fatigue of bearing in mind the numbers was thus obviated. The numbers obtained were taken to

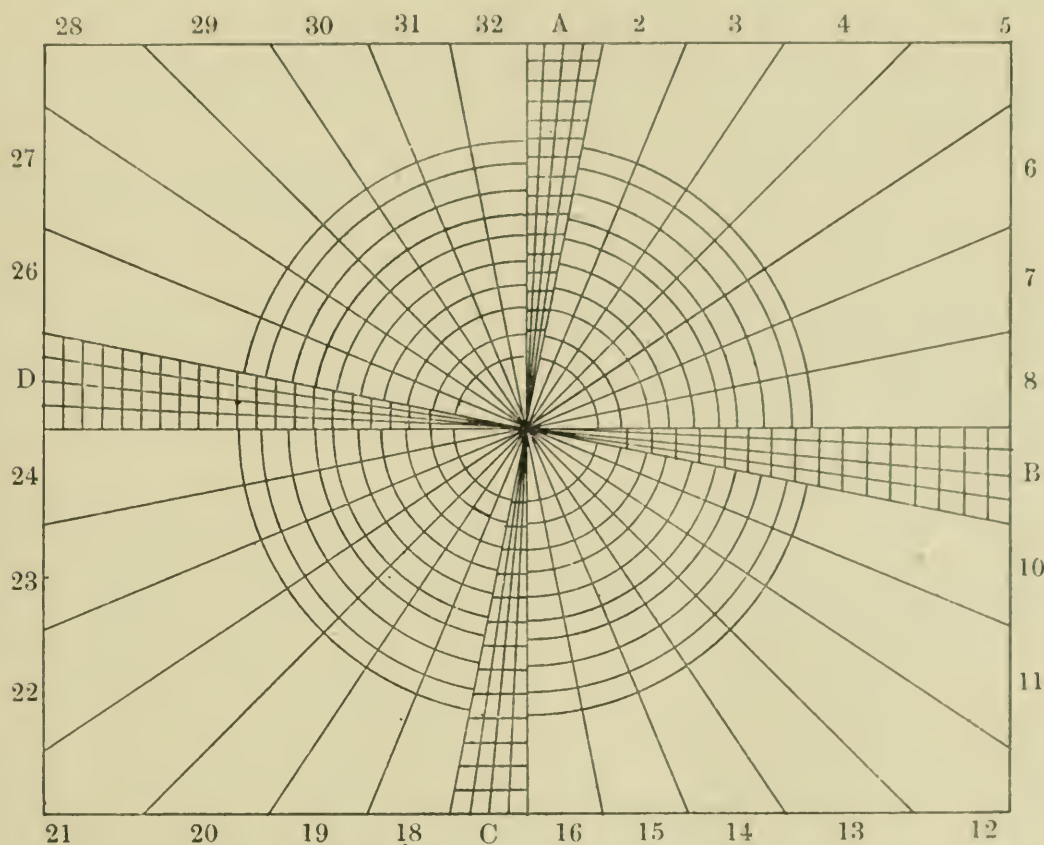


Fig. 1. Counting Plate. ( $\times \frac{1}{2}$ .)

indicate the number of living organisms capable of growth present in a standard loopful of the culture at the commencement of the experiment.

The standard loop with the wire slightly bent was dipped into the fluid to a certain depth when the tube was tilted towards the horizontal. When used in this manner the loop was found to carry 0.01 c.c.

After varying periods of incubation the cultures were violently shaken to distribute the organisms and standard loopfuls removed. When considerable growth was indicated by slight cloudiness of the medium dilution was found to be necessary before plating. In most cases the loopful was diluted in 5 or 10 c.c. of sterile distilled water, and from this after violent shaking a loopful was transferred to agar at 45° C. The plate cultures thus obtained often

contained very numerous colonies, which, if the manipulations had been carefully performed, were very evenly distributed. In such cases the colonies in at least four of the thirty-two sections into which the ruled counting plate was divided, or an area equivalent to one-eighth of the whole plate, were counted. The sections marked *A*, *B*, *C*, *D* in Fig. 1, subdivided to ensure greater accuracy in counting, were chosen. The numbers counted in these four sections multiplied by eight and the factor for dilution, were taken to represent the living organisms in one loopful of the culture.

Throughout the paper the figures represent the numbers calculated to be present in a standard loopful or 0.01 c.c. of the cultures.

*Sources of error in these experiments.*

There are several possible sources of error in these experiments of which the most important seem to be the following:

(1) Uneven distribution of the organisms in the culture (see Section XVIII) at the time of taking the sample due to insufficient agitation of the culture before taking the samples or uneven distribution in the diluting fluid due to insufficient agitation before plating. On various occasions several plates were made from the same cultures after different periods of shaking and gave remarkably similar results. It seems probable, therefore, that if the cultures and the dilutions are sufficiently shaken errors from this cause are not of great importance.

(2) Uneven distribution of the organisms owing to unequal breaking up of the clumps formed in the process of multiplication. For varying periods after division organisms such as cocci remain united, and a single colony may represent the descendants of one, two, three, four or perhaps more individuals. Errors from this cause are unavoidable in all experiments of this type. Microscopic examination of the fluid of the culture after shaking usually showed well separated cocci mixed with occasional small clumps consisting of three or four individuals. Also the deep colonies in any given plate were usually of very similar size, apparently indicating that growth had occurred from single organisms or very small clumps. Spontaneous agglutination is said to occur in old cultures. No evidence of the occurrence of this phenomenon was obtained.

(3) Variations in the quantity taken up by the loop. The quantity of fluid taken up by the same loop on different occasions varies, but with careful manipulation the variations are not sufficient to influence materially the broad conclusions. Most of the experiments were carried out in duplicate, a procedure which tends to correct errors due to variations in the quantity carried by the loop. The figures quoted are in many cases the means of readings from two plate cultures made from separate dilutions.

(4) Death of the organisms in the diluting fluid. In all cases cultures were made from the diluting fluid within a few minutes. As almost uniform



results were obtained in cultures made from the diluting fluid at intervals up to 30 minutes this possible source of error may be neglected.

(5) Excessive numbers of colonies on the plates. When very high numbers were suspected plates were prepared from several different dilutions so as to obtain some plates containing suitable numbers for counting.

(6) Contamination of the cultures. When contaminating organisms developed in the cultures the experiments were discontinued.

(7) The failure of living organisms to produce visible colonies within the period of incubation. Plates were counted on several occasions after one, two and three days' incubation, and almost invariably gave approximately identical figures. Only in very exceptional circumstances did the colonies become more numerous after two days' incubation. In most cases twenty-four hours may be considered a sufficient period of incubation.

It is evident that with the many sources of error involved in such experiments results of great accuracy cannot be expected, but the events occurring in the cultures are indicated broadly.

#### *Method of describing the experiments.*

Each series of experiments is considered in the following way. First the aim of the experiment is explained, next the composition of the media used and methods employed are stated and then the results are given in the form of tables and charts showing the numbers of organisms calculated as present per standard loop of the culture on each occasion of counting. Finally the sequence of events presumably occurring in the culture is indicated and the general conclusions which appear to be permissible.

Irregularities in the curves have been regarded as due in many cases to errors in manipulation.

#### SECTION I. *Growth in neutral meat extract medium.*

Several experiments have been carried out at different times in the two years during which the work has been in progress to ascertain the course of events in neutral meat extract cultures of a certain strain of *S. aureus*. In different experiments, provided that the cultures used for inoculating the meat extract had been grown under similar conditions and the number of organisms inoculated was small, very similar results were obtained.

Since the results vary according to the numbers inoculated and the previous history of the culture it is impossible to establish an exact standard with which the results of other experiments can be compared. Consequently every series of experiments devised to ascertain the effects of altered conditions should be accompanied by controls.

In order to keep the strains of organisms alive and vigorous "stock" cultures were grown on meat extract agar, and subcultivated every 8-10 days. Previous to each experiment a subculture was made on the surface of agar and incubated for 18 hours at 37° C. From this subculture an emulsion

in distilled water was prepared, and one drop of the emulsion was used for inoculating each tube containing meat extract 1 c.c., *N*/10 soda 0.08 c.c.<sup>1</sup>, distilled water 3.92 c.c. The emulsions were of such strength that each loopful of the medium after inoculation contained less than 10,000 organisms. Immediately after inoculation and the distribution of the organisms by violent shaking a standard loopful of the culture was added to melted agar at 45° C. and a plate culture made in order to ascertain the number of organisms present at the commencement of the experiment. After various periods of incubation at 37° C. standard loopfuls were transferred to distilled water (usually 5 or 10 c.c.), and from these dilutions plate cultures were prepared in the manner previously described. The results of four experiments carried out in November and December 1918 are quoted, and Chart 1 is constructed

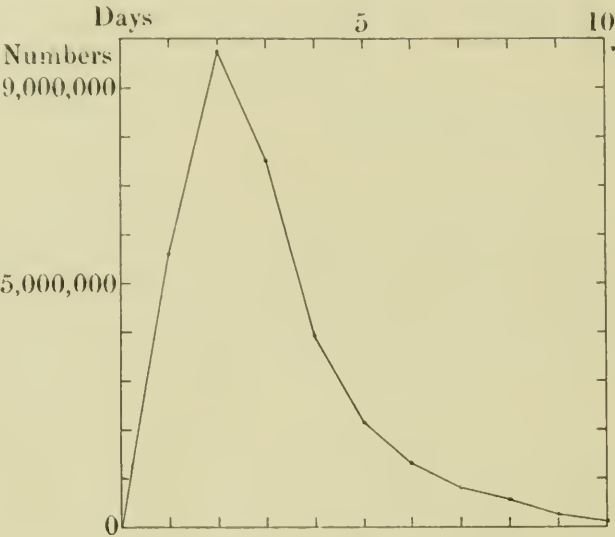


Chart 1. Illustrating the numbers of living cocci present daily in a neutral meat extract culture.

from the mean daily counts. This chart may be taken to illustrate the course of events in a culture of *Staphylococcus aureus* under the conditions described<sup>2</sup>, and further it may be used to some extent as a rough standard.

	A	B	C	D	Mean
Original numbers:	1784	5660	1392	520	2339
Days. 1	7,632,000	7,680,000	6,208,000	5,920,000	6,610,000
2	9,280,000	9,872,000	10,606,000	9,248,000	9,751,000
3	6,656,000	5,840,000	8,656,000	8,905,000	7,514,000
4	2,992,000	4,016,000	3,120,000	5,632,000	3,940,000
5	2,312,000	2,560,000	1,600,000	2,280,000	2,188,000
6	1,608,000	1,376,000	872,000	1,376,000	1,308,000
7	688,000	732,000	724,000	1,080,000	806,000
8	—	492,000	508,000	704,000	568,000
9	—	148,000	200,000	466,000	271,000
10	—	48,000	68,000	216,000	111,000
11	—	27,500	32,000	78,000	46,000
12	—	9,984	—	—	—

<sup>1</sup> It was found that in most preparations of meat extract *N*/10 soda to the extent of 0.08 c.c. to each 1 c.c. of meat extract was required to bring the fluid to the neutral point of neutral red.

<sup>2</sup> The results of the inoculation of different numbers and of organisms grown under other conditions are given elsewhere (Sections IV and V).



Multiplication proceeds very rapidly during the first day and more slowly in the second, when the maximum number, about 10,000,000 per standard loop, is reached. Later the number of living organisms decreases at first rapidly, but later more slowly, until a low level is reached, which remains fairly constant for a long period, in spite of the diminution of the volume of the fluid by evaporation. In some instances cultures have been examined after 53 days' incubation, when the volume of the fluid had decreased to less than 1 c.c. Even under these circumstances large numbers of the organisms were found to be alive, apparently indicating that the survivors were little influenced by the increasing concentration of their products brought about by evaporation. During this prolonged period of relative constancy in numbers small oscillations are observed in all experiments of this nature. To determine whether such oscillations are the result of faulty technique or are due to occasional periods of slight multiplication and subsequent diminution in numbers would require further investigation.

SECTION II. *The influence of the previous rate of transplantation of the culture used for inoculation.*

The frequency with which the cultures used for inoculation have been subcultivated on agar slopes influences the results sufficiently to modify the appearance of the charts. Three observations are quoted to illustrate this influence.

Experiment (I) was similar in all respects with those quoted in the last section except that the cocci used for inoculation had been transplanted at intervals of three or four days during a period of two months. Experiment (II) was the same in all respects as experiment (I), but was carried out nine months later. Experiment (B) was similar except that the cocci used were transplanted daily for six days.

		Exp. (I)	Exp. (II)	Mean of (I) and (II)	Exp. (B)
Original numbers:		632	5920	3176	5760
Days.	1	5,968,000	6,928,000	6,448,000	9,696,000
	2	8,744,000	8,112,000	8,428,000	12,976,000
	3	10,448,000	10,240,000	10,344,000	11,248,000
	4	9,968,000	9,056,000	9,487,000	2,864,000
	5	8,688,000	7,104,000	7,896,000	—
	6	7,002,000	4,096,000	5,549,000	1,244,000
	7	2,496,000	2,192,000	2,344,000	408,000
	8	852,000	1,592,000	1,222,000	272,000
	9	296,000	1,092,000	646,000	148,000
	10	252,000	114,000	186,000	79,500
	11	212,000	70,000	141,000	46,500
	12	204,000	56,000	130,000	13,824
	13	87,500	42,000	64,000	29,056
	14	61,500	24,500	42,100	30,400
	15	44,500	—	—	—
	16	37,500	—	—	—
	17	17,000	—	—	—
	18	14,460	—	—	—

Frequent transplantation over a long period (Expts. I, II) results in the maximum being reached a day later than in the standard, and in the line of decline in numbers lagging about two days behind the standard. Several subcultivations in rapid succession (Expt. B) result in very rapid growth, a high maximum and a very rapid fall in the numbers.

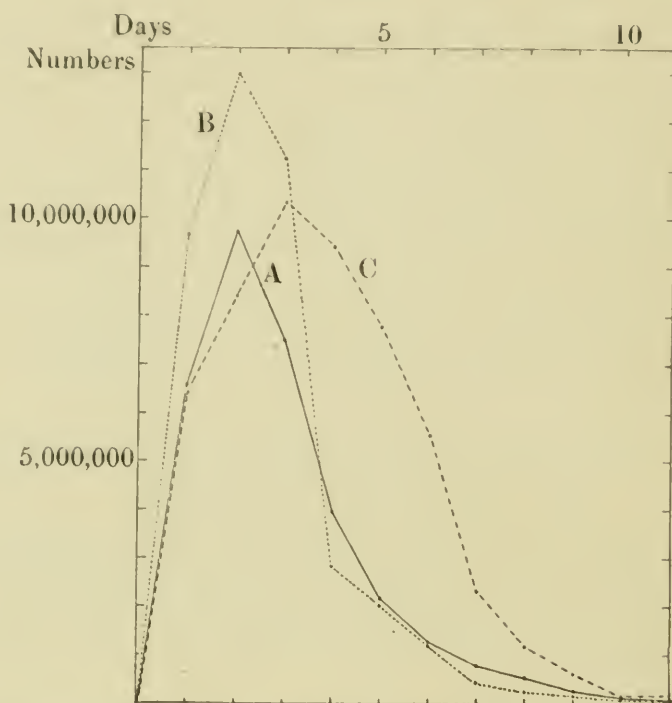


Chart 2. Showing deviations from the "standard" curve, A. Curve B illustrates the type of growth when a culture which has been rapidly transplanted and curve C when a culture which has been subcultivated frequently over a long period is used.

### SECTION III. *The relation of growth to the quantity of food substance present.*

In order to ascertain the relation of growth to the proportion of food substance (meat extract) present, five tubes containing the following ingredients were each inoculated with a drop of an emulsion of *Staphylococcus aureus* and incubated at 37° C.

Tube	Meat extract	Distilled water
1	5 c.c.	0 c.c.
2	3.75 c.c.	1.25 c.c.
3	2.5 "	2.5 "
4	1.25 "	3.75 "
5	.5 "	4.5 "

Before incubation the mean number of cocci per standard loopful in each tube was 868.

	22 hrs.	40 hrs.	64 hrs.	88 hrs.	112 hrs.	138 hrs.	162 hrs.
Tube 1	11,808,000	18,960,000	23,424,000	25,840,000	22,816,000	19,376,000	16,384,000
2	10,664,000	16,960,000	20,300,000	20,864,000	17,936,000	13,784,000	9,840,000
3	8,880,000	13,288,000	15,760,000	13,696,000	10,960,000	6,904,000	2,448,000
4	6,296,000	9,336,000	9,416,000	5,736,000	732,000	218,000	118,000
5	3,566,000	4,273,000	3,392,000	568,000	57,500	17,750	17,750



	186 hrs.	204 hrs.	234 hrs.	258 hrs.	282 hrs.	306 hrs.	330 hrs.
Tube 1	14,832,000	13,976,000	11,520,000	10,664,000	9,008,000	6,044,000	2,920,000
2	7,713,000	6,984,000	3,920,000	1,024,000	362,000	378,000	204,000
3	992,000	334,000	78,000	23,000	41,000	28,750	26,000
4	68,750	31,750	13,750	12,000	16,250	11,500	10,000
5	15,500	16,750	56,500	57,000	57,750	76,000	71,750
	354 hrs.	372 hrs.	396 hrs.	420 hrs.	444 hrs.	468 hrs.	26 days
Tube 1	1,352,000	446,000	434,000	300,000	182,000	284,000	63,808
2	44,000	32,000	81,000	124,000	127,000	326,000	170,496
3	39,000	84,000	—	140,000	—	142,000	62,848
4	13,750	28,000	26,250	27,500	—	8,464	24,168
5	—	—	—	20,750	—	1,152	736

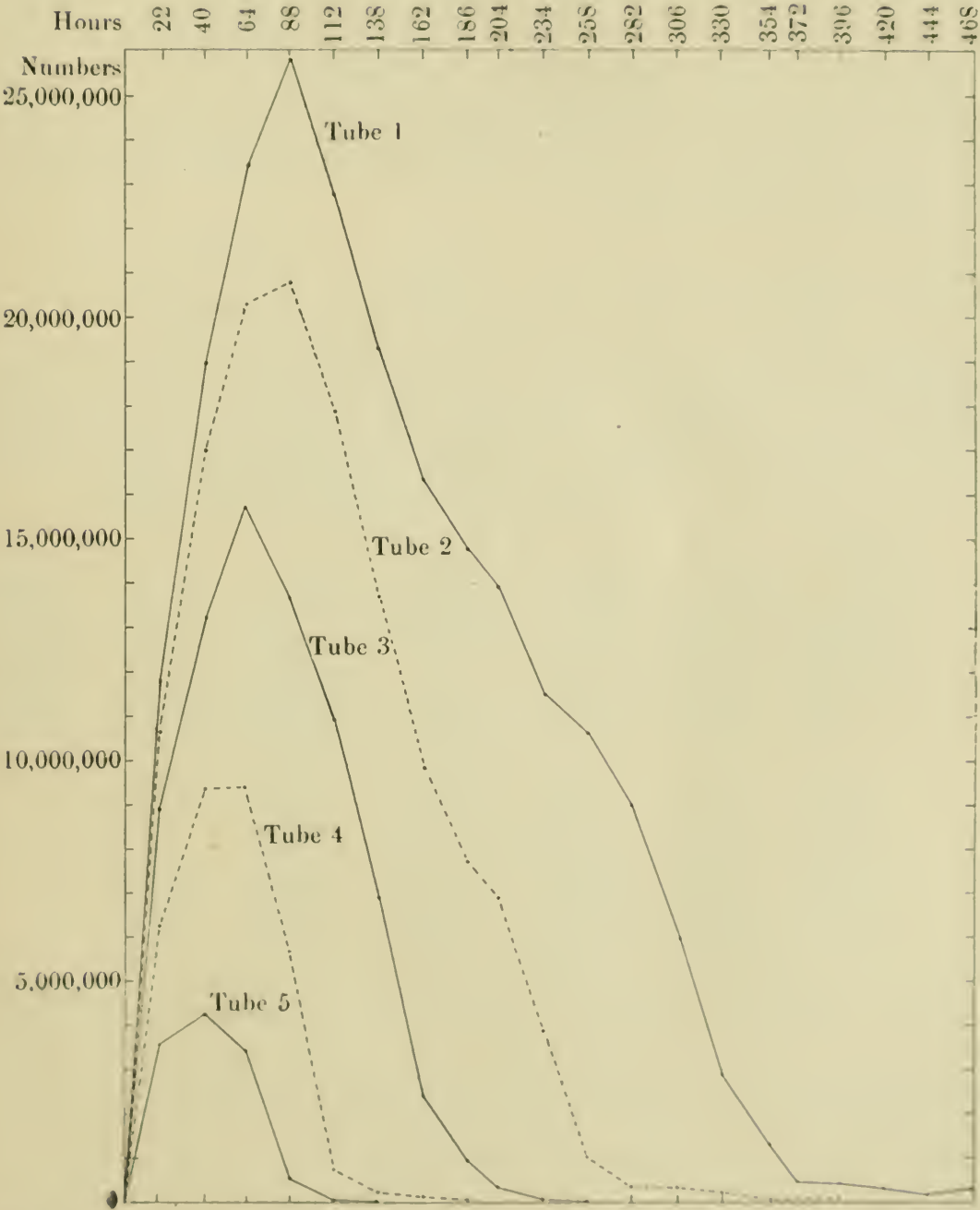


Chart 3. Showing the relation of growth to the proportion of food substance present. Tube 1 contains the largest proportion and tubes 2, 3, 4 and 5 contain  $\frac{3}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{4}$  and  $\frac{1}{16}$  respectively of the amount in tube 1.

	53 days *	88 days	144 days	209 days
Tube 1	253,725	24,768	29,500	11,744
2	146,816	4,256	12,000	15,360
3	48,896	3,392	4,000	8,576
4	102,912	6,784	32,000	8,768
5	720	360	1,000	128

\* Contents of tubes reduced to 1 c.c. or less by evaporation. On 60th, 110th, 121st and 156th days made up to 5 c.c. with distilled water.

It is evident that the greater the proportion of meat extract the greater is the multiplication and the longer the period which elapses before the curve reaches its highest point; in fact the multiplication appears to be proportional to the concentration of meat extract in the culture. The length of the period of rapid decline is also related to the concentration of the meat extract.

After the period of rapid decline small numbers of the organisms remain alive for an indefinite time. As in other experiments slight fluctuations in their numbers seemed to occur.

#### SECTION IV. *The influence of the numbers inoculated.*

In order to ascertain the extent to which the course of events at 37° C. is influenced by considerable differences in the numbers of cocci inoculated several experiments were carried out of which one is quoted. Each tube contained meat extract 1 c.c., *N*/10 soda 0.08 c.c., distilled water 3.92 c.c.

	Original number	20 hrs.	2 days	3 days	4 days	5 days	6 days
No. 1	25,664,000	27,616,000	28,696,000	22,368,000	15,872,000	8,112,000	1,296,000
2	9,248,000	13,024,000	13,344,000	10,152,000	7,552,000	5,128,000	2,552,000
3	3,216,000	11,566,000	12,848,000	—	5,920,000	2,624,000	936,000
4	1,392	6,208,000	10,606,000	8,656,000	3,120,000	1,600,000	872,000
5	520	5,920,000	9,248,000	8,905,000	5,632,000	2,280,000	1,376,000
	7 days	8 days	9 days	10 days	11 days	23 days	
No. 1	488,000	152,000	71,000	41,500	47,500	5	
2	2,112,000	1,552,000	1,344,000	800,000	504,000	18,584	
3	824,000	728,000	608,000	300,000	248,000	14,376	
4	724,000	508,000	200,000	68,000	32,000	16,356	
5	1,080,000	704,000	566,000	216,000	78,000	8,704	

In this medium with a small inoculation the maximum number of cocci present at any period does not usually exceed 10–12 millions per standard loop. If the initial dose greatly exceeds this figure multiplication proceeds relatively slowly for two days and subsequently there is a very rapid fall in numbers. With an initial dose close to this figure a somewhat similar curve is produced, though the rate of fall is not so rapid. Much smaller initial doses produce the “standard” type of curve.



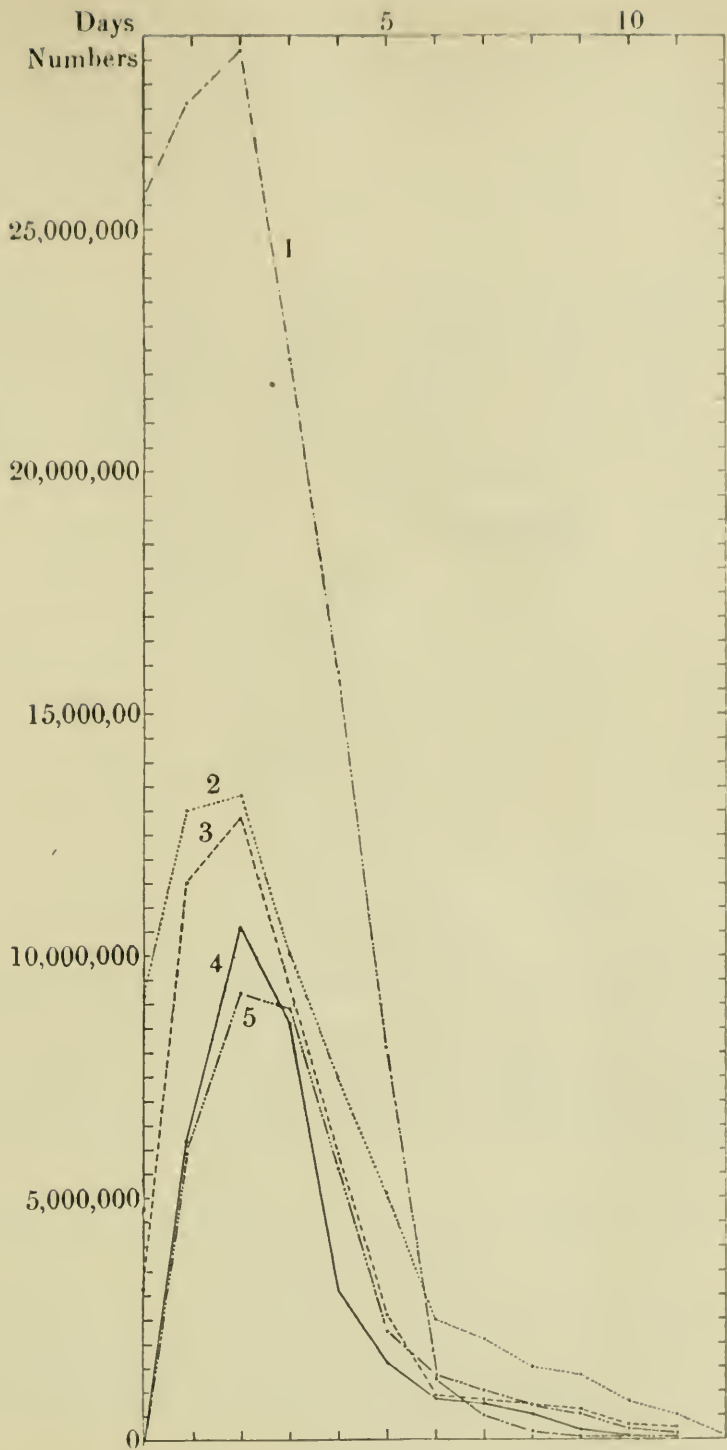


Chart 4. Showing the influence of the dose of organisms inoculated into the medium. Tube 1 contained at the beginning 25 million cocci per loop; tube 2, 9 million; tube 3, 3 million; tube 4, 1392; and tube 5, 520.

SECTION V. *The influence of small differences in numbers in the initial inoculation.*

In most of the experiments quoted in this paper small drops of dilute emulsions of *S. aureus* were used for inoculating the cultures. The experiments of Section V were carried out in order to ascertain to what extent small differences in the numbers inoculated influence counts made after 24 or more

hours' cultivations at 37° C. An emulsion of moderate strength was first made, and from this dilutions calculated to contain approximately  $\frac{1}{10}$ ,  $\frac{1}{100}$  and  $\frac{1}{1000}$  of the organisms. It will be seen from the table that after 26 hours' cultivation all four cultures contained nearly equal numbers. By mistake a slightly greater proportion than usual of water was added to the meat extract.

	Original number	2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	26 hrs.
Full strength	34,400	56,800	694,000	2,748,000	4,544,000	6,250,000	8,720,000
$\frac{1}{10}$	4,300	6,200	12,750	210,000	1,408,000	3,840,000	8,544,000
$\frac{1}{100}$	420	928	1,775	9,300	34,600	471,000	8,496,000
$\frac{1}{1000}$	59	101	320	2,090	9,760	49,000	7,584,000

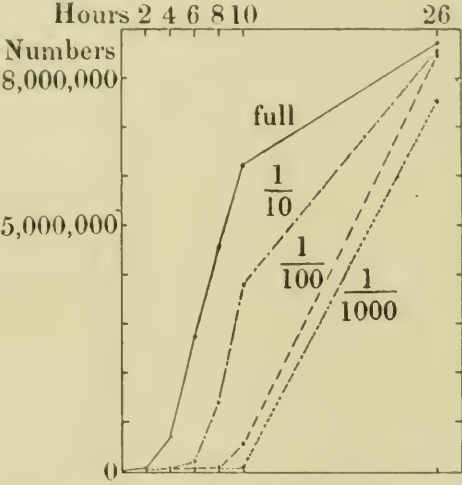


Chart 5. Showing the rate of growth in cultures inoculated with small numbers of cocci.

The variations in numbers usually encountered in emulsions made in the manner generally employed in these experiments are not likely therefore to affect very materially the results in different experiments of the same kind.

SECTION VI. *The influence of occasional additions of small quantities of food substance.*

Seeing that the concentration of products by evaporation appeared to have little effect on the surviving organisms, a series of experiments, of which one is quoted, was undertaken to ascertain the results of adding fresh food material at different stages of cultivation.

Four tubes, *A*, *B*, *C*, *D*, containing meat extract .75 c.c., *N*/10 soda 0.06 and distilled water 4.19 c.c. were inoculated with drops of an emulsion of *S. aureus*, and the numbers estimated daily in the usual manner. The tubes were incubated at 37° C. The mean number of cocci per standard loopful at the beginning of the experiment was 816.

In the case of tube *A* no addition was made up to the 12th day and the curve up to that time follows the normal course. On the 12th day ten drops of undiluted meat extract were added resulting in a great rise in the numbers, followed by a gradual decline. On the 23rd day when again a low level had



been reached two drops of concentrated meat extract (40 c.c. of meat extract evaporated to 1.0 c.c. at 40° C.) were added, and resulted in a rise and subsequent fall in the numbers somewhat similar to that which occurred at the beginning of the experiment.

This experiment appears to show that the fall in numbers is due mainly to exhaustion of food material, and not to accumulation of products.

In the case of tube *B* a drop of undiluted meat extract was added on the 4th and 5th days when the number was declining and caused a retardation in the rate of the fall. On the 10th day six drops of meat extract were added and two drops on each of the following three days. This procedure resulted in a moderate rise like that seen in tube *A*. On the 23rd day when a low level had been reached two drops of the concentrated meat extract given to tube *A* were added, and gave a similar result. On the 37th day the volume of the culture had fallen to 0.53 c.c. owing to evaporation, and sufficient sterile distilled water was added to bring up the volume to 5 c.c. On the 40th day one drop of the concentrated meat extract was added and resulted in a rise in numbers followed by a slow decline. On the 56th and three following days a drop of concentrated meat extract was added and caused a very great increase in the numbers. The experiment was abandoned on the 59th day.

In tubes *C* and *D* growth was allowed to proceed till the 4th day when a decline in numbers was beginning to occur. In the case of tube *C* a drop of meat extract of the same composition as the medium in the tube was added daily from the 4th to the 9th days. This resulted in a slow but steady decline showing that insufficient nutrient material was being supplied daily to keep the numbers at a high level. From the 10th to the 22nd days two drops were added daily, resulting in the rate of decline becoming slower. An irregularity in the curve of unknown causation occurred on the 15th, 16th and 17th days.

In tube *D* a drop of distilled water was added from the 4th to the 9th days and two drops from the 10th to the 23rd days. This slight dilution caused no obvious deviation from the usual course of events.

On the 23rd day two drops of the concentrated meat extract supplied to tubes *A* and *B* were added to *C* and *D* and resulted in considerable rises followed by rapid declines. The rise of *D* was higher than that of *C* and may perhaps be accounted for by some difference in the quantity of material added. Again on the 40th day one drop of the same concentrated meat extract was added to each tube and resulted in a rise followed by a decline. The rise in *C* was greater than in *D*. After a sufficient decline in numbers had taken place a drop of concentrated meat extract (50 c.c. of meat extract evaporated to 2.0 c.c. at 40° C.) was added daily to each tube between the 56th and 64th days, and resulted in a great daily rise in the numbers for five days. Then for four days the numbers remained very high, but with a tendency to remain at approximately the same level. The oscillations in the chart were probably due to irregularities in the drops added and to difficulties in counting such large numbers. On the 64th and 65th days smaller drops

were used. In the case of tube *C* the experiment ceased at this point, but in the case of tube *D* it was continued the smaller drops being added daily by means of a glass tube passed through the cork of the vessel containing the meat extract. In this way drops of approximately equal size were delivered. This procedure resulted in an initial fall in the numbers, and then in the numbers remaining approximately the same for each day's count. The experiment was discontinued on the 75th day.

On the 37th day owing to evaporation each tube contained less than 5 c.c. of fluid. They were made up to 5 c.c. with sterile distilled water.

Tube	Immediate culture	1 day	2 days	3 days	4 days
<i>A</i>	816	4,864,000	5,872,000	6,928,000	5,100,000
<i>B</i>	—	5,024,000	6,000,000	6,696,000	5,168,000 + 1*
<i>C</i>	—	4,016,000	6,160,000	6,352,000	5,264,000 + 1
<i>D</i>	—	4,272,000	6,112,000	6,664,000	4,992,000 + 1 w.
	5 days	6 days	7 days	8 days	9 days
<i>A</i>	3,072,000	742,000	372,000	280,000	140,000
<i>B</i>	4,536,000 + 1	3,408,000	1,054,000	248,000	120,000
<i>C</i>	4,949,000 + 1	4,496,000 + 1	3,848,000 + 1	2,928,000 + 1	2,476,000 + 1
<i>D</i>	3,260,000 + 1 w.	536,000 + 1 w.	344,000 + 1 w.	208,000 + 1 w.	— + 1 w.
	10 days	11 days	12 days	13 days	14 days
<i>A</i>	171,000	75,500	60,500 + 10	2,812,000	2,572,000
<i>B</i>	140,000 + 6	1,786,000 + 2	2,212,000 + 2	2,504,000 + 2	1,984,000
<i>C</i>	2,416,000 + 2	2,200,000 + 2	1,968,000 + 2	1,696,000 + 2	1,740,000 + 2
<i>D</i>	160,000 + 2 w.	82,500 + 2 w.	64,000 + 2 w.	59,000 + 2 w.	77,500 + 2
	15 days	16 days	17 days	18 days	19 days
<i>A</i>	2,088,000	1,056,000	696,000	528,000	312,000
<i>B</i>	600,000	300,000	160,000	104,000	64,000
<i>C</i>	2,008,000 + 2	1,264,000 + 2	1,236,000 + 2	729,000 + 2	780,000 + 2
<i>D</i>	180,000 + 2 w.	102,000 + 2 w.	43,500 + 2 w.	49,500 + 2 w.	32,000 + 2 w.
	20 days	21 days	22 days	23 days	24 days
<i>A</i>	140,000	92,000	65,000	53,000 + 2 c.	7,232,000
<i>B</i>	—	96,000	—	45,000 + 2 c.	5,536,000
<i>C</i>	628,000 + 2	976,000 + 2	1,200,000 + 2	1,304,000 + 2 c.	5,264,000
<i>D</i>	43,500 + 2 w.	224,000 + 2 w.	96,000 + 2 w.	75,500 + 2 c.	7,600,000
	25 days	26 days	27 days	28 days	29 days
<i>A</i>	8,448,000	—	8,336,000	4,536,000	1,720,000
<i>B</i>	6,960,000	7,024,000	8,288,000	3,568,000	1,552,000
<i>C</i>	5,520,000	5,448,000	5,280,000	4,320,000	1,632,000
<i>D</i>	8,112,000	7,888,000	8,440,000	3,536,000	1,472,000
	30 days	31 days	32 days	33 days	34 days
<i>A</i>	1,008,000	742,000	544,000	338,000	254,000
<i>B</i>	1,064,000	888,000	—	436,000	356,000
<i>C</i>	1,502,000	840,000	488,000	292,000	332,000
<i>D</i>	1,312,000	1,240,000	1,008,000	800,000	536,000

\* The sign + 1 indicates that a drop of meat extract was added immediately after the sample for estimating the numbers was taken, + 1 c. indicates a drop of concentrated meat extract, and + 1 w. indicates a drop of sterile distilled water.



Tube	35 days	36 days	37 days	39 days	40 days
<i>A</i>	271,000	228,000	made up to 5 c.c.	12,864	1 drop concentrated meat extract added
<i>B</i>	344,000	276,000	„	8,192	„
<i>C</i>	304,000	360,000	„	12,496	„
<i>D</i>	548,000	380,000	„	3,744	„
	41 days	42 days	43 days	44 days	45 days
<i>A</i>	—	—	—	—	—
<i>B</i>	2,512,000	5,552,000	4,128,000	3,712,000	3,560,000
<i>C</i>	3,936,000	5,280,000	4,928,000	5,008,000	3,768,000
<i>D</i>	3,352,000	2,666,000	2,312,000	2,672,000	2,664,000
	46 days	47 days	48 days	49 days	50 days
<i>A</i>	—	—	—	—	—
<i>B</i>	2,320,000	1,992,000	1,840,000	1,715,000	1,312,000
<i>C</i>	2,096,000	2,448,000	2,072,000	2,112,000	1,408,000
<i>D</i>	2,512,000	1,744,000	416,000	116,000	22,500
	51 days	52 days	53 days	54 days	55 days
<i>A</i>	—	—	—	—	—
<i>B</i>	1,128,000	680,000	556,000	245,000	72,000
<i>C</i>	1,048,000	488,000	268,000	132,000	148,000
<i>D</i>	39,000	70,500	74,000	56,000	12,000
	56 days	57 days	58 days	59 days	60 days
<i>A</i>	—	—	—	—	—
<i>B</i>	49,700 + 1 c.	2,472,000 + 1	9,744,000 + 1	13,792,000 + 1	—
<i>C</i>	43,950 + 1 c.	3,880,000 + 1	11,568,000 + 1	17,360,000 + 1	19,488,000 + 1
<i>D</i>	1,670 + 1 c.	3,584,000 + 1	10,272,000 + 1	17,968,000 + 1	19,152,000 + 1
	61 days	62 days	63 days	64 days	65 days
<i>C</i>	— + 1	24,736,000 + 1	23,892,000 + 1	25,744,000 + 1 s.*	26,118,000 + 1 s.
<i>D</i>	— + 1	26,176,000 + 1	24,160,000 + 1	23,840,000 + 1 s.	25,472,000 + 1 s.
	66 days	67 days	68 days	69 days	70 days
<i>C</i>	22,448,000 + 1 s.	—	—	—	—
<i>D</i>	20,768,000 + 1 s.	— + 1 s.	16,136,000 + 1 s.	14,506,000 + 1 s.	14,064,000 + 1 s.
	71 days	72 days	73 days	74 days	75 days
<i>D</i>	13,232,000	13,008,000	12,850,000	12,960,000	12,736,000

\* + 1 s. indicates a smaller drop than previously used. See text, p. 148.

This experiment shows that in the medium used the rapid fall in numbers which follows the initial rise is not due to the accumulation of products, but is caused mainly by the using up of food material, since the addition at any time of small quantities of fresh food material, insufficient in amount to cause appreciable dilution of the products, results in further growth to some extent proportional to the amount of food material added. Moreover it shows that very small daily additions of food material retard the rate of decline and that large daily additions cause a great rise in the numbers, resulting in a high and approximately constant level being maintained at least for some time.

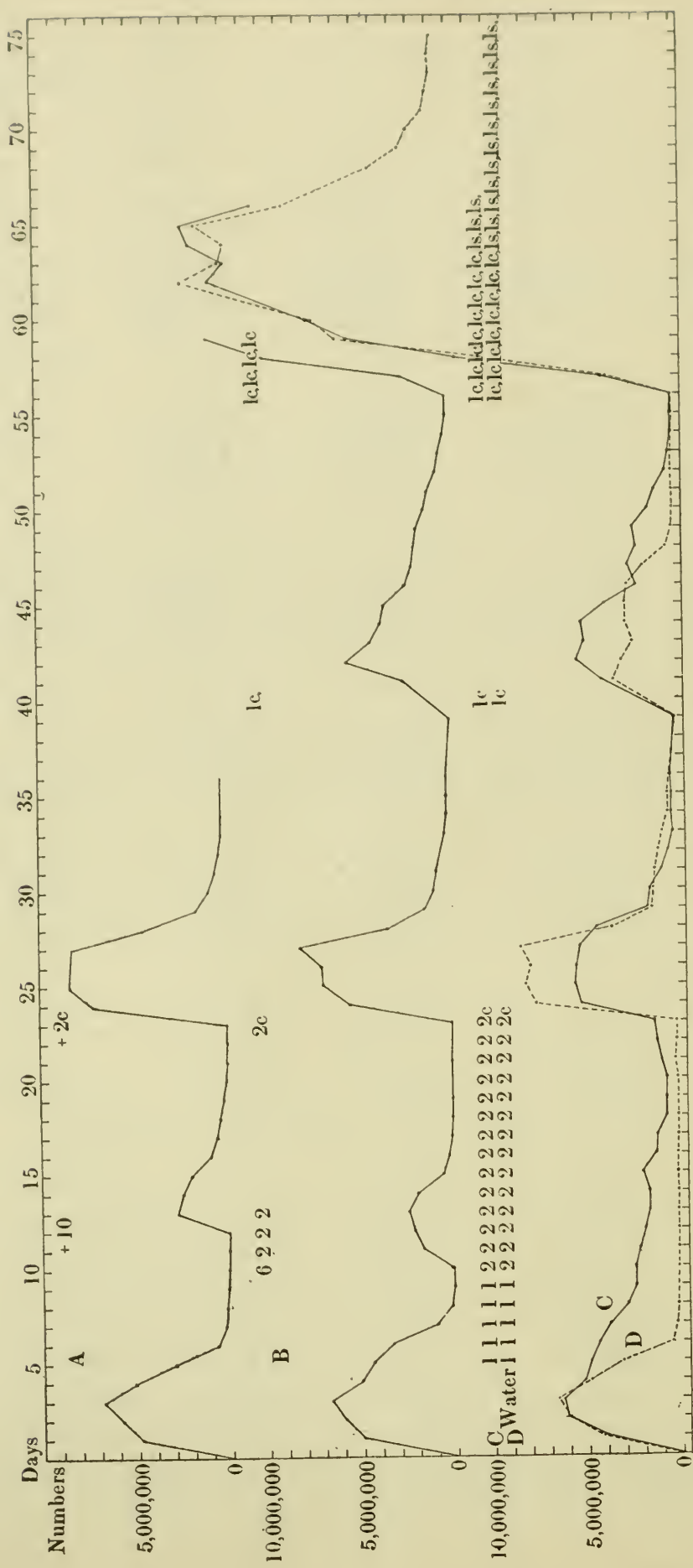


Chart 6. Showing the effects of occasional small additions of food substance to meat extract cultures. + 10 = 10 drops of meat extract added; 2c = 2 drops of concentrated meat extract added; 1s = 1 small drop of concentrated meat extract from a special pipette added. In tube D drops of water were added.



SECTION VII. *The influence of regular additions of small quantities of food substance.*

This experiment was carried out in order to ascertain whether it is possible to keep the numbers of bacteria in a culture incubated at 37° C. at a constant level by daily additions of small quantities of food material.

In each of three large test-tubes, *A*, *B* and *C*, 10 c.c. of sterile distilled water were placed. To each two drops of a concentrated meat extract (50 c.c. of meat extract evaporated to 7 c.c. at 40° C.) were added daily up to the 13th day. Approximate uniformity in the size of the drops was secured by keeping the concentrated meat extract in a test-tube closed with a paraffined cork through which a glass tube, drawn out at its inner end, passed. Drops from this tube were used throughout the experiment. Immediately after the first addition of meat extract each tube was inoculated with a drop of an emulsion of *S. aureus*.

The numbers increased daily for five days and then kept approximately level at about 9,000,000 per loop up to the 14th day. On and after that date one drop only was added daily. The numbers fell rapidly during the next three days to 2·5 millions per loop and then remained at this level till the conclusion of the experiment on the 27th day.

Chart 7 illustrating this experiment is compiled from the daily mean of the three tubes *A*, *B* and *C* up to the 12th day, when tube *A* became contaminated; from the daily mean of tubes *B* and *C* to the 21st day, when tube *B* became contaminated. The counts were made, except on the occasion stated in the table, at the same time each day.

Tube	Original number	30 hrs.	2 days	3 days	4 days	5 days
<i>A</i>	3808	2,576,000	3,376,000	4,544,000	6,624,000	9,088,000
<i>B</i>	3136	2,736,000	3,248,000	4,128,000	6,702,000	8,832,000
<i>C</i>	3516	2,272,000	3,904,000	4,976,000	7,024,000	9,744,000
Mean	3487	2,528,000	3,509,000	4,549,000	6,786,000	9,221,000
9 days, 1 hr. earlier than usual						
	6 days	7 days	8 days	10 days	11 days	
<i>A</i>	9,888,000	9,992,000	9,136,000	9,744,000	10,000,000	10,200,000
<i>B</i>	8,736,000	8,528,000	8,960,000	8,656,000	8,656,000	8,576,000
<i>C</i>	10,184,000	9,496,000	9,112,000	9,776,000	9,818,000	9,600,000
Mean	9,603,000	9,305,000	9,069,000	9,392,000	9,491,000	9,459,000
	12 days	13 days	14 days*	15 days	16 days	17 days
<i>A</i>	9,640,000	—	—	—	—	—
<i>B</i>	8,232,000	8,878,000	8,608,000	8,464,000	6,208,000	2,344,000
<i>C</i>	9,221,000	8,976,000	9,024,000	9,136,000	6,336,000	2,952,000
Mean	9,031,000	8,927,000	8,811,000	8,800,000	6,272,000	2,648,000
	18 days	19 days	20 days	21 days	22 days	23 days
<i>A</i>	—	—	—	—	—	—
<i>B</i>	2,456,000	2,432,000	2,720,000	2,624,000	—	—
<i>C</i>	—	2,040,000	1,924,000	1,984,000	2,240,000	2,368,000
Mean	2,456,000	2,236,000	2,322,000	2,304,000	—	—
	24 days	25 days	26 days	27 days		
<i>C</i>	2,456,000	2,420,000	2,272,000	2,448,000		

\* 1 drop only of concentrated meat extract added on and after this day.

By subcultures made at short intervals on certain days it was shown that after each addition of food material the numbers increase for a few hours and then decrease. Consequently estimations made every twenty-four hours indicate the general effect of the additions.

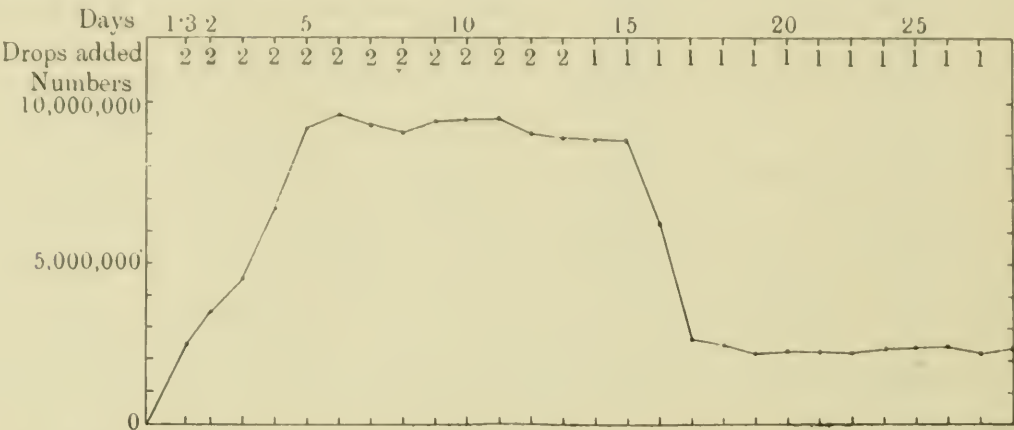


Chart 7. Showing the effects of regular small additions of food substance to meat extract cultures.

It is evident from this experiment that by suitable small regular additions of food material a concentration of *Staphylococci* within certain desired limits could be maintained in such a fluid culture medium for a long period of time. Accumulation of the products may gradually inhibit growth, but on this point the experiment gives little evidence.

SECTION VIII. *The effect of diluting cultures with distilled water at various periods of growth.*

In one series of experiments three tubes *A*, *B*, *C* each containing meat extract 1 c.c., *N*/10 soda 0.08 c.c. and distilled water 3.92 c.c. were inoculated with a drop of an emulsion of a *Staphylococcus* culture and incubated at 37° C. At the commencement of the experiment about 4400 cocci were present per standard loopful. After 24 hours' incubation the numbers in each tube were counted. Then 1 c.c. of distilled water was added to tube *B*, and 2.5 c.c. to tube *C* and the numbers present in each estimated immediately. Tube *A* was used as a control. The cultures were incubated at 37° C. for 11 days and counts made daily.

A second similar series of tubes *D*, *E*, *F* made from another sample of meat extract were incubated for four days. On that date after the numbers present in each tube had been counted 1 c.c. of sterile distilled water was added to tube *E* and 2.5 c.c. to tube *F*, and the numbers present in each estimated immediately. These cultures were incubated subsequently for seven days and counts made daily.

Tube	Number present originally	1 day before dilution	After dilution	2 days	3 days
<i>A</i>	4,400	5,440,000	—	9,772,000	11,344,000
<i>B</i>	—	5,195,000	4,944,000	8,176,000	9,664,000
<i>C</i>	—	5,456,000	3,376,000	6,880,000	8,256,000
<i>D</i>	—	5,840,000	—	9,600,000	10,960,000
<i>E</i>	—	5,600,000	—	9,088,000	10,552,000
<i>F</i>	—	5,744,000	—	9,854,000	10,528,000



	4 days				
Tube	before dilution	After dilution	5 days	6 days	7 days
A	10,032,000	—	6,686,000	5,744,000	4,496,000
B	8,912,000	—	5,600,000	4,720,000	3,488,000
C	6,880,000	—	4,656,000	3,216,000	2,720,000
D	6,944,000	—	3,288,000	2,772,000	2,048,000
E	6,784,000	5,504,000	2,384,000	2,144,000	1,584,000
F	6,480,000	4,784,000	2,176,000	1,772,000	1,048,000
	8 days	9 days	10 days	11 days	
A	3,088,000	2,488,000	1,048,000	560,000	
B	2,944,000	2,256,000	788,000	204,000	
C	1,696,000	1,280,000	588,000	272,000	
D	844,000	520,000	256,000	66,500	
E	612,000	432,000	170,000	47,000	
F	568,000	196,000	—	—	

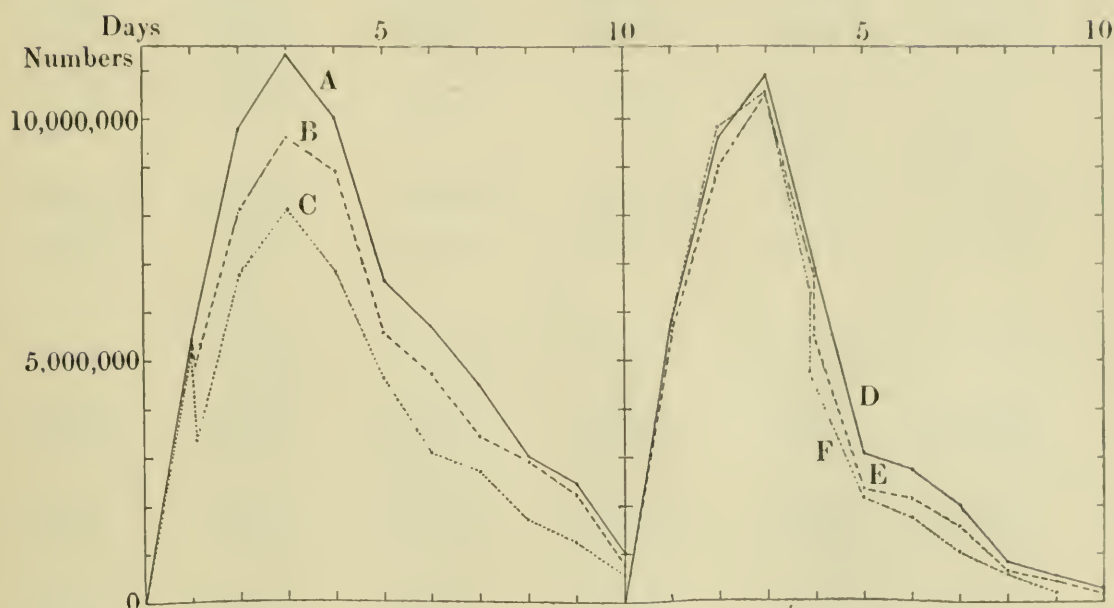


Chart 8. Showing the effects of diluting cultures at different times during incubation.

Though some of these cultures have been diluted no food material has been removed, and each culture contains the same amount of food at different dilutions. If growth goes on as usual each tube should contain at any given time the same number of organisms, though in each standard loopful the number should be proportional to the dilution. This seems to be the case and it may be concluded that moderate dilution with distilled water at any stage of incubation has little effect on multiplication.

#### SECTION IX. *The influence of different incubation temperatures on the growth of S. aureus.*

In the first of the experiments quoted three tubes, A, B, C, each containing meat extract 1 c.c., N/10 soda 0.08 c.c. and distilled water 3.92 c.c. were sterilised by boiling, and inoculated with a drop of an emulsion of a *Staphylococcus* culture, which had been transplanted frequently. Tube A was cultivated

at 37° C., tube *B* at 27° C. and tube *C* at 17·0° C. Daily counts were made for the first 18 days, but subsequently only tube *C* was counted daily.

Tube	Number originally present		1 day	2 days	3 days	4 days
<i>A</i> at 37° C.	632		5,968,000	8,744,000	10,448,000	9,968,000
<i>B</i> at 27° C.	—		5,528,000	10,918,000	12,568,000	14,288,000
<i>C</i> at 17° C.	—		2,064	73,850	3,120,000	8,128,000
	5 days	6 days	7 days	8 days	9 days	10 days
<i>A</i>	8,688,000	7,002,000	2,496,000	852,000	296,000	152,000
<i>B</i>	15,918,000	15,664,000	13,568,000	11,488,000	7,248,000	3,584,000
<i>C</i>	12,400,000	14,512,000	17,072,000	18,272,000	17,592,000	16,304,000
	11 days	12 days	13 days	14 days	15 days	16 days
<i>A</i>	212,000	204,000	87,500	61,000	44,500	37,500
<i>B</i>	1,896,000	1,286,000	906,000	432,000	516,000	480,000
<i>C</i>	16,176,000	16,200,000	16,128,000	15,988,000	15,760,000	15,256,000
	17 days	18 days	19 days	20 days	21 days	22 days
<i>A</i>	17,000	14,460	—	—	—	11,424
<i>B</i>	444,000	360,000	—	—	—	—
<i>C</i>	14,096,000	13,664,000	12,000,000	11,488,000	10,592,000	10,134,000
	23 days	24 days	25 days	26 days	27 days	28 days
<i>A</i>	—	—	—	—	6,016	—
<i>B</i>	—	—	—	—	39,040	—
<i>C</i>	9,664,000	9,324,000	8,192,000	8,064,000	6,304,000	6,544,000
	29 days	30 days	31 days	32 days	33 days	34 days
<i>C</i>	5,816,000	5,912,000	5,535,000	4,464,000	3,456,000	2,656,000
	35 days	36 days	37 days	38 days	39 days	40 days
<i>C</i>	2,056,000	1,704,000	1,452,000	1,160,000	1,090,000	976,000
	41 days	42 days	43 days	44 days	45 days	46 days
<i>C</i>	976,000	914,000	844,000	662,000	295,000 *	262,000

\* Culture made up to 5 c.c. with distilled water.

It will be seen that the highest maximum was attained and the numbers remained greatest for the longest period in the culture grown at 17° C., and the least maximum was attained and the numbers fell most quickly in the culture grown at 37° C. As this was contrary to expectations a similar experiment was carried out six months later.

In this case six cultures were prepared. Two of them were incubated at 33° C., two at 27° C. and two at 8 to 10° C. The culture used for inoculation had not been so frequently transplanted as the one used in the experiment just quoted. For the cultures grown at 33° C. and 27° C. the figures given in the table represent the mean for the two tubes used at each temperature, but the results of each of the tubes incubated at 8 to 10° C. are quoted separately, since the results in the two tubes were somewhat different. After the 26th day the results in one only of the tubes incubated at 33° C. are quoted to illustrate the fluctuations which sometimes occur at this period (see p. 141).



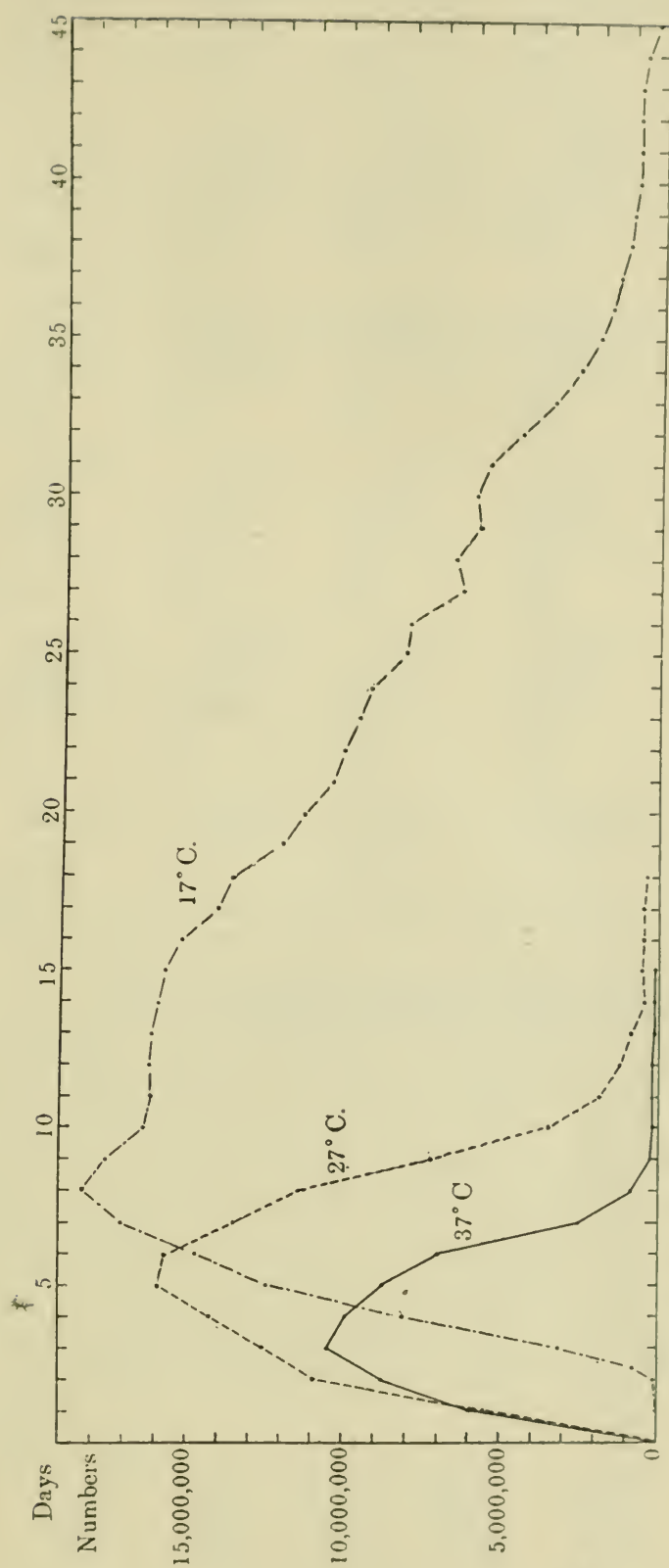


Chart 9. Showing the results of incubating meat extract cultures at 37° C., 27° C. and 17° C.

Number originally present		1 day	2 days	3 days	4 days	5 days
Culture at 33° C.	2024	7,360,000	9,632,000	10,288,000	10,610,000	6,410,000
"	27° C. —	6,528,000	13,104,000	17,647,000	18,671,000	18,184,000
"	(1) 8-10° C. —	2,300	1,904	1,728	1,592	1,448
"	(2) " —	2,304	2,104	—	2,016	1,984
		6 days	7 days	8 days	9 days	10 days
Culture at 33° C.	2024	4,880,000	3,704,000	2,280,000	1,536,000	914,000
"	27° C. —	15,968,000	15,488,000	16,072,000	12,712,000	6,680,000
"	(1) 8-10° C. —	1,240	1,152	936	802	536
"	(2) " —	1,712	1,648	1,392	1,336	1,236
		11 days	12 days	13 days	14 days	15 days
Culture at 33° C.	2024	870,000	693,000	558,000	496,000	154,000
"	27° C. —	5,946,000	3,525,000	2,365,000	2,412,000	2,012,000
"	(1) 8-10° C. —	398	297	312	253	262
"	(2) " —	1,062	997	940	939	—
		17 days	19 days	21 days	23 days	26 days
Culture at 33° C.	2024	65,500	9,744	4,192	6,744	24,448*
"	27° C. —	1,312,000	834,000	932,000	612,000	—
"	(1) 8-10° C. —	164	155	120	54	64
"	(2) " —	752	728	440	379	290†
		28 days	31 days	35 days	39 days	41 days
Culture at 33° C.	2024	10,016	4,032	15,424	19,328	74,500
"	27° C. —	276,000	360,000	260,000	270,000	165,000
"	(1) 8-10° C. —	14	26	21	19	—
"	(2) " —	281	136	47	47	26
		44 days	47 days	52 days	65 days	
Culture at 33° C.	2024	65,000	53,500	10,500	—	
"	27° C. —	—	—	—	62,800	
"	(1) 8-10° C. —	—	—	—	14	
"	(2) " —	7	—	—	—	

\* One culture only quoted on and after this date.

† Culture contaminated by a mould.

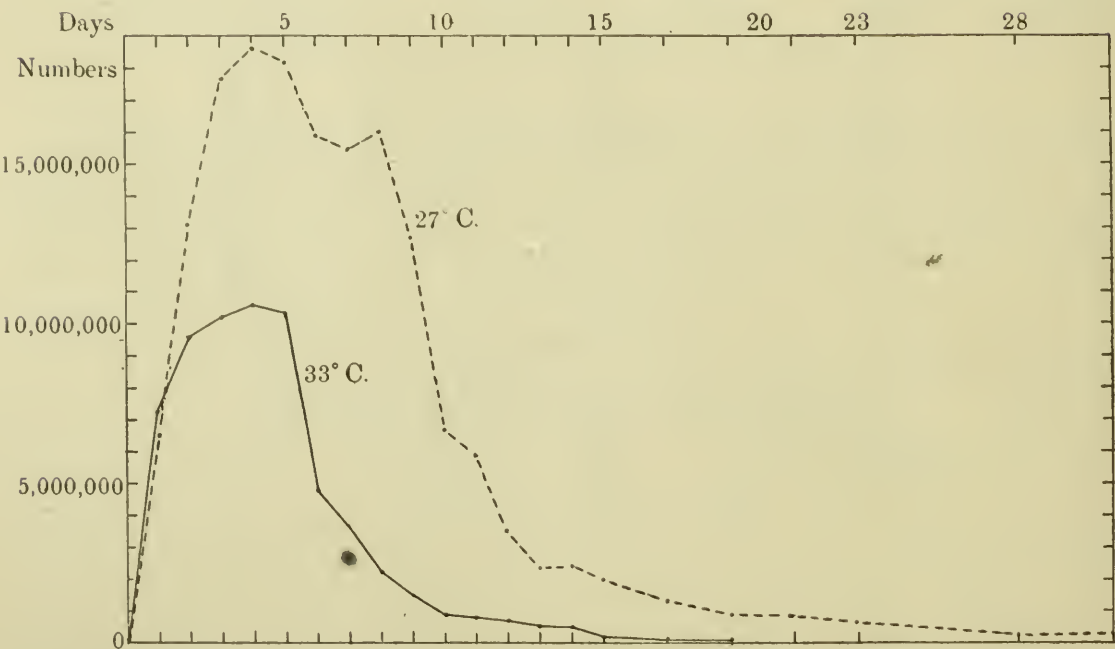


Chart 10. Showing the results of incubating meat extract cultures at 33° C. and 27° C.



The results obtained with cultures grown at 33° C. and at 27° C. confirm the results of the first experiment.

If we regard the rate of multiplication within the first few days as indicating the specially favourable conditions for growth then a temperature between  $37^{\circ}\text{C.}$  and  $27^{\circ}\text{C.}$  may be regarded as the most suitable for the growth of the *S. aureus*. On the other hand if we regard the maximum numbers attained at any period of incubation as indicating the most favourable conditions then a temperature about  $17^{\circ}\text{C.}$  may be regarded as the most suitable for the growth of the coccus.

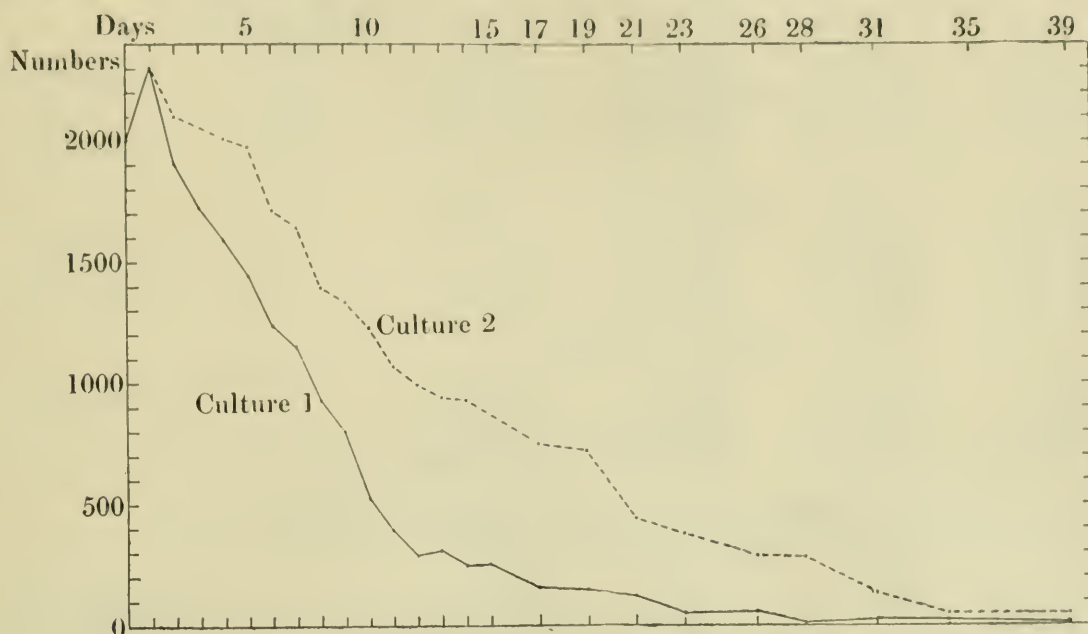


Chart 11. Showing the daily counts in two cultures incubated at 8 to 10° C.

At 8 to 10° C. very slight multiplication, if any, occurs during the first 24 hours' incubation and subsequently the numbers steadily decline for at least 60 days. The experiment was not continued long enough to decide whether at this temperature the organisms ultimately die out.

This unexpected result led to the carrying out of a series of experiments at lower temperatures. Two tubes of the same medium inoculated with *S. aureus* were incubated at about  $-1^{\circ}\text{C}.$ , two at about  $-6^{\circ}\text{C}.$ , and two at  $-10^{\circ}$  to  $-12^{\circ}\text{C}.$ , and the numbers counted daily. The mean for the two tubes kept at each temperature is recorded in the tables. Occasionally some of the tubes were frozen. These were thawed very slowly before samples were taken for counting.

[illegible]

From the 21st to the 28th days all the tubes were incubated at 37° C. but no signs of growth occurred and no organisms were found on subculture. It may therefore be assumed that the cultures had completely died out.

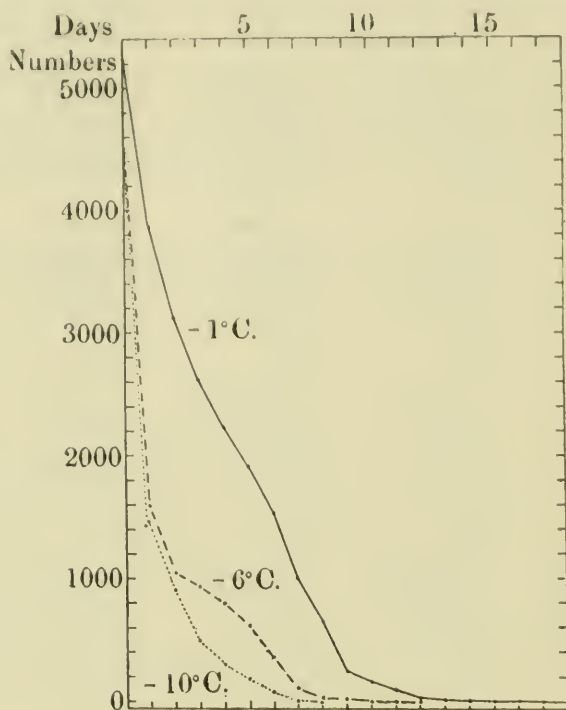


Chart 12. Showing the results of keeping meat extract cultures at low temperatures, -1° C., -6° C. and -10° C.

It will be seen that in each culture the numbers rapidly declined and eventually died out. At -10° C. the cocci were dead on the 9th day, at -6° C. on the 13th day and at -1° C. on the 19th day.

It seems possible that at some temperature between 10° C. and 17° C. the numbers might remain approximately constant.

#### SECTION X. *The influence of the growth of one species on the growth of others subsequently inoculated into the medium.*

Some of the experiments described in previous sections appear to indicate that the fall in numbers is due mainly to the exhaustion of the food supply. The following experiments were undertaken in the hope of throwing some further light on this subject. 21 tubes each containing meat extract 1 c.c., N/10 soda 0.08 c.c. and water 3.92 c.c. were prepared in the usual manner. Seven of the tubes were inoculated with an emulsion of *S. aureus*, seven with an emulsion of *B. coli*, and seven with an emulsion of *B. pyocyaneus*. All these cultures were incubated at 37° C. for sixteen days, and during that time from one culture of each organism, acting as a control, counts were made daily in order to ascertain the course of events in each series. At the end of that time a slight daily fall in numbers was occurring in each culture, and it was presumed that the greater part of the food supply had been exhausted.



On the 16th day the six cultures of each organism were divided into two groups, *A* and *B*, three cultures in each group. Those of group *A* were not sterilised, but those of group *B* were sterilised by boiling. Then a drop of an emulsion of *S. aureus* was added to cultures *A*<sub>1</sub> and *B*<sub>1</sub>, a drop of an emulsion of *B. coli* to cultures *A*<sub>2</sub> and *B*<sub>2</sub>, and a drop of an emulsion of *B. pyocyaneus* to cultures *A*<sub>3</sub> and *B*<sub>3</sub> of each series, and the number of organisms present in each culture immediately estimated. Daily counts were made for the next seven days to ascertain to what extent multiplication of the added organisms took place.

	Number originally present	1 day	2 days	3 days	4 days
Staphylococci ...	5920	6,928,000	8,112,000	10,240,000	9,056,000
<i>B. coli</i> ...	3408	3,217,000	6,256,000	11,712,000	9,440,000
<i>B. pyocyaneus</i> ...	3232	13,568,000	24,784,000	18,252,000	18,206,000
		5 days	6 days	7 days	8 days
Staphylococci ...		7,104,000	4,096,000	2,192,000	1,592,000
<i>B. coli</i> ...		5,216,000	3,504,000	2,232,000	2,032,000
<i>B. pyocyaneus</i> ...		11,808,000	10,528,000	9,536,000	7,664,000
		9 days	10 days	11 days	12 days
Staphylococci ...		1,096,000	114,500	70,000	56,000
<i>B. coli</i> ...		1,968,000	1,312,000	1,247,000	896,000
<i>B. pyocyaneus</i> ...		6,416,000	6,288,000	6,016,000	6,592,000
		13 days	14 days	15 days	16 days
Staphylococci ...		42,000	24,500	25,000	21,000
<i>B. coli</i> ...		944,000	688,000	512,000	316,000
<i>B. pyocyaneus</i> ...		6,272,000	5,040,000	—	4,896,000

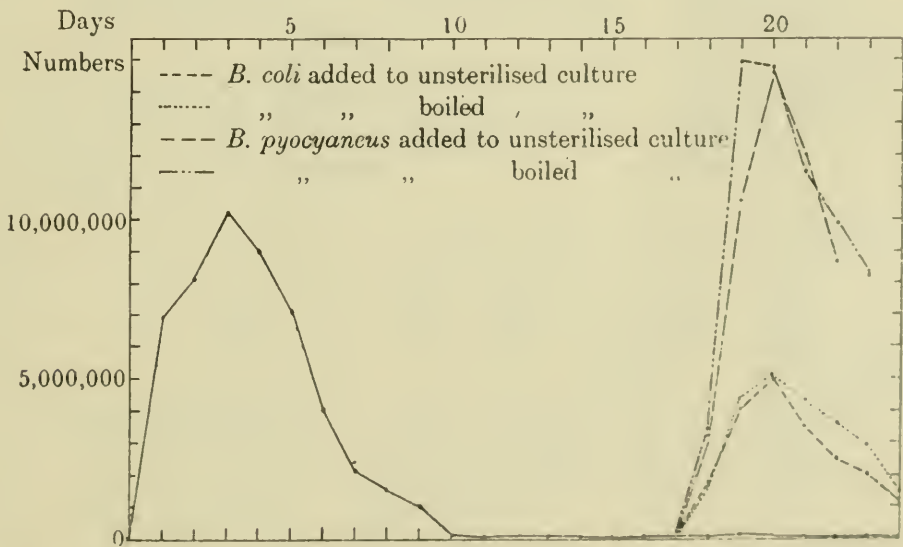


Chart 13. Showing the rate of multiplication of added organisms in meat extract in which *S. aureus* had been growing for 17 days.

In the sterilised groups (*B*) multiplication of the added bacteria took place in every case, whether the added organisms belonged to the species previously growing in the medium or not. After a culture had been sterilised

by boiling some food was therefore available for freshly added organisms, even though they belonged to the same species and strain.

In the unsterilised groups (*A*) multiplication of the added bacteria took place in all cases with two exceptions, when *B. coli* and *B. pyocyaneus* were added respectively to cultures in which the same species had been growing.

It is difficult to compare with any degree of accuracy the extent of multiplication in comparable examples of the two groups, *A* and *B*, since in the

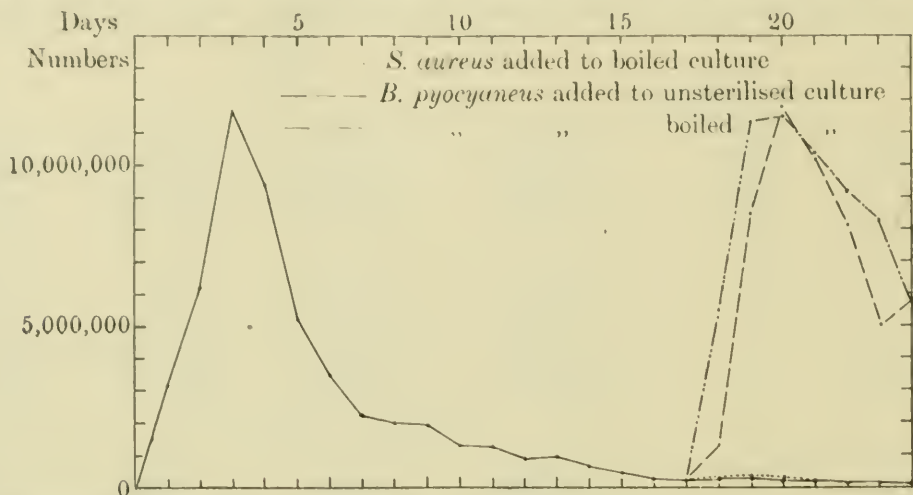


Chart 14. Showing the rate of multiplication of added organisms in tubes in which *B. coli* had been growing for 17 days.

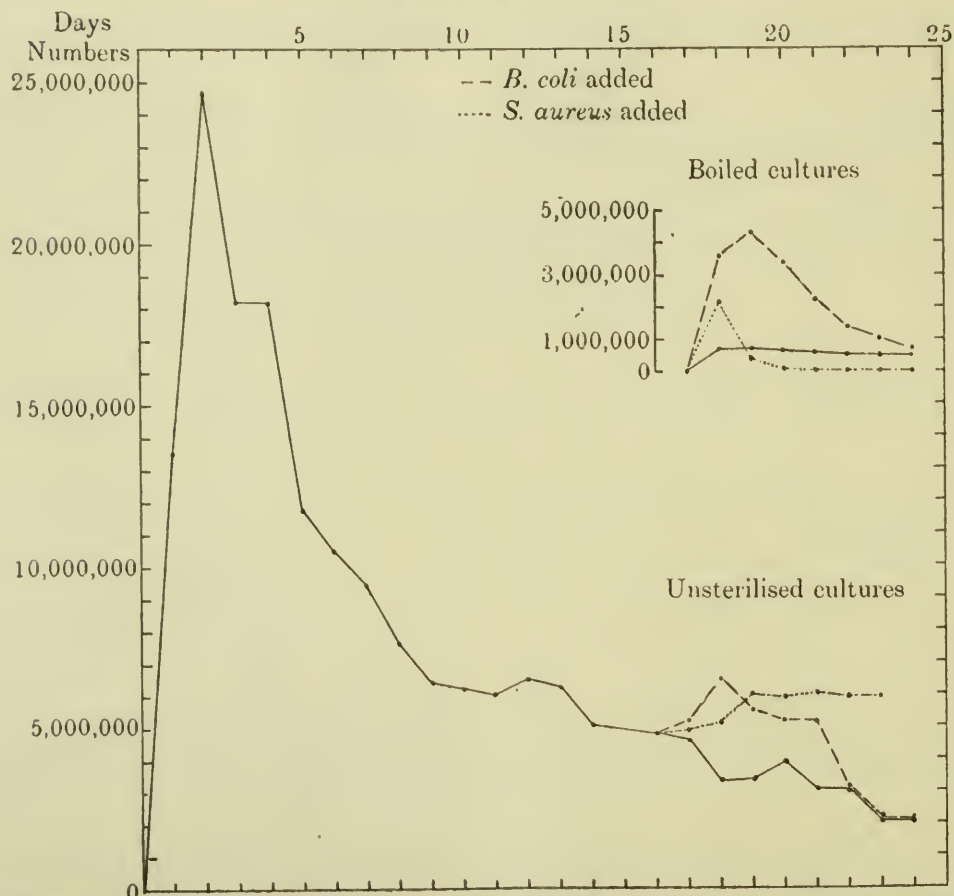


Chart 15. Showing the rate of multiplication of added organisms in tubes in which *B. pyocyaneus* had been growing for 17 days.



		17th day after inoculation	18th day	19th day	20th day	21st day	22nd day	23rd day	24th day
COCCUS CULTURE									
Unsterilised	Coccus added $A_1$	24,000	53,000	111,500	59,500	28,500	21,800	20,184	16,128
	" " $A_2$	27,000	1,840,000	4,096,000	4,992,000	3,536,000	2,464,000	2,096,000	1,168,000
	<i>B. pyocyaneus</i> " $A_3$	29,000	3,248,000	10,688,000	14,656,000	12,048,000	8,640,000	8,208,000	6,616,000
Sterilised	Coccus added $B_1$	2,560	44,500	95,000	32,500	2,000	1,008	1,872	2,296
	" " $B_2$	3,072	1,824,000	4,400,000	5,024,000	4,384,000	3,600,000	2,912,000	1,560,000
	<i>B. pyocyaneus</i> " $B_3$	6,144	3,424,000	14,896,000	14,720,000	11,520,000	9,984,000	8,320,000	—
<i>B. COLI</i> CULTURE									
Unsterilised	Coccus added $A_1$	452,000	492,000	648,000	668,000	580,000	576,000	416,000	376,000
	" " $A_2$	288,000	312,000	256,000	188,000	172,000	71,500	41,856	21,366
	<i>B. pyocyaneus</i> " $A_3$	324,000	1,392,000	8,524,000	11,808,000	10,144,000	8,144,000	5,008,000	5,876,000
Sterilised	Coccus added $B_1$	2,176	172,000	212,500	134,000	19,500	1,664	536	—
	" " $B_2$	2,912	296,000	328,000	296,000	280,000	328,000	208,000	39,425
	<i>B. pyocyaneus</i> " $B_3$	6,048	5,584,000	11,328,000	11,584,000	10,448,000	9,264,000	8,320,000	5,760,000
<i>B. PYOCYANEUS</i> CULTURE									
Unsterilised	Coccus added $A_1$	4,972,000	5,136,000	6,080,000	5,984,000	6,080,000	5,936,000	5,968,000	—
	" " $A_2$	5,232,000	6,513,000	5,568,000	5,288,000	5,248,000	3,376,000	3,248,000	3,008,000
	<i>B. pyocyaneus</i> " $A_3$	4,640,000	3,312,000	3,424,000	3,968,000	3,088,000	3,072,000	2,062,000	2,096,000
Sterilised	Coccus added $B_1$	2,312	2,200,000	440,000	27,500	16,500	6,350	4,704	5,728
	" " $B_2$	2,784	3,624,000	4,320,000	3,488,000	2,320,000	1,424,000	1,040,000	728,000
	<i>B. pyocyaneus</i> " $B_3$	5,888	712,000	744,000	656,000	628,000	576,000	—	576,000

unsterilised series, *A*, a gradual decline in the numbers of the organisms originally present was doubtless proceeding, and the counting of controls, which would have shown the extent of the decline during this period of the experiment, was not continued after the 16th day. A rough comparison was obtained in the following manner. In each example of the sterilised group of cultures, *B*, the highest figure recorded was divided by the number introduced, while in comparable examples of the unsterilised group, *A*, the number at the time the fresh organisms were added (group *B*, column 1, 17th day, subtracted from comparable culture, group *A*, column 1) was subtracted from the highest figure recorded and the result divided by the number introduced. The figures so obtained indicate very roughly the extent to which multiplication took place in each case.

Cultures originally inoculated with	Multiplication of added		
	<i>S. aureus</i>	<i>B. coli</i>	<i>B. pyocyaneus</i>
<i>S. aureus</i>	(unsterilised, <i>A</i> <sub>1</sub> ) × 35	( <i>A</i> <sub>2</sub> ) × 1617	( <i>A</i> <sub>3</sub> ) × 2381
	(sterilised, <i>B</i> <sub>1</sub> ) × 37	( <i>B</i> <sub>2</sub> ) × 1635	( <i>B</i> <sub>3</sub> ) × 2424
<i>B. coli</i>	(unsterilised, <i>A</i> <sub>1</sub> ) × 100	( <i>A</i> <sub>2</sub> ) × 8?	( <i>A</i> <sub>3</sub> ) × 1916
	(sterilised, <i>B</i> <sub>1</sub> ) × 97	( <i>B</i> <sub>2</sub> ) × 112	( <i>B</i> <sub>3</sub> ) × 1915
<i>B. pyocyaneus</i>	(unsterilised, <i>A</i> <sub>1</sub> ) × 480	( <i>A</i> <sub>2</sub> ) × 461	( <i>A</i> <sub>3</sub> ) × 0
	(sterilised, <i>B</i> <sub>1</sub> ) × 951	( <i>B</i> <sub>2</sub> ) × 1551	( <i>B</i> <sub>3</sub> ) × 126

These figures seem to indicate that the *Staphylococci* added to tubes of media, whether sterilised or unsterilised, in which the same organism had been growing multiplied to a small and nearly equal extent; when added to sterilised or unsterilised tubes in which *B. coli* had been growing multiplication in both cases was about three times as great, and when added to tubes in which *B. pyocyaneus* had been growing multiplication was greater, 14 times as great in the unsterilised and 27 times in the sterilised.

*B. coli* added to tubes, sterilised or unsterilised, in which *S. aureus* had been growing multiplied to a considerable and equal extent, and it multiplied to nearly the same extent in the sterilised tube in which *B. pyocyaneus* had been growing, but to only one-quarter of the extent in the unsterilised tube. When it was added to the unsterilised tube in which *B. coli* had been growing there was little or no multiplication, but when added to the sterilised tube moderate multiplication occurred.

*B. pyocyaneus* multiplied to a great and equal extent in sterilised or unsterilised tubes in which *S. aureus* had been growing and to a less, but equal extent, in sterilised or unsterilised tubes in which *B. coli* had been growing. In the unsterilised tube in which *B. pyocyaneus* had been growing there was no multiplication, but there was moderate multiplication in the sterilised tube.

It will be noticed that when *S. aureus* or *B. coli* had been growing in the medium boiling in either case did not increase the food value of the medium for the other two species. The food value for added *S. aureus* was not increased by boiling a culture in which *S. aureus* had been growing, though in the case



of *B. coli* the food value of the medium for added *B. coli* seemed to be increased by boiling the medium in which that organism had been growing. On the other hand after the growth of *B. pyocyaneus* boiling caused added organisms to grow more freely than they did on the unsterilised medium.

The growth of any of these organisms in the medium seems to remove a portion of the food substance used by other species since in no case was the growth of an added species nearly so considerable in extent as in its primary culture. If it is assumed that the increase in numbers is proportional to the food supply the growth of *Staphylococcus* removes about half the food originally available for *B. coli* and for *B. pyocyaneus*; the growth of *B. coli* removes most of the food available for *Staphylococci*, and about half of that available for *B. pyocyaneus*.

Cocci added to unsterilised coccus cultures appear to make use of some material, which those already present do not seem to have utilised. This does not appear to be the case when *B. coli* or *B. pyocyaneus* are added to their own cultures.

The results obtained in the experiments just quoted were controlled by another series of experiments.

Sixty tubes each containing meat extract 1 c.c., *N/10* soda 0.08 c.c. and distilled water 3.92 c.c. were prepared. Twenty were inoculated with *S. aureus*, twenty with *B. coli*, and twenty with *B. pyocyaneus* and incubated at 35° C. Of each series two were incubated for one day, two for two days, two for three days, two for five days and two for seven days. One of each of the pairs just mentioned was boiled, the other not sterilised, and to each 4 c.c. of 2 per cent. agar in distilled water at 50° C. were added, and plates poured. After the medium had set three streaks of heavy emulsions of *S. aureus*, *B. coli* and *B. pyocyaneus* were made across each plate, and the results recorded after 48 hours' incubation at 37° C.

#### Cultures in which *S. aureus* was growing.

Period of growth	Unsterilised series			Boiled series		
	Streaks of cocci	<i>B. coli</i>	<i>B. pyocyaneus</i>	Cocci	<i>B. coli</i>	<i>B. pyocyaneus</i>
7 days	0	++	++	0	++	++
5 „	0	++	++	*?	++	++
3 „	0	++	++	+	++	++
2 „	0	++	++	+	++	++
1 day	*?	++	++	+	++	++

#### Cultures in which *B. coli* was growing.

Period of growth	Unsterilised series			Boiled series		
	Streaks of cocci	<i>B. coli</i>	<i>B. pyocyaneus</i>	Cocci	<i>B. coli</i>	<i>B. pyocyaneus</i>
7 days	0	0	++	*	++	++
5 „	0	0	++	+	++	++
3 „	*?	0	++	+	++	++
2 „	*?	0	++	+	++	++
1 day	*?	0	++	+	++	++

Cultures in which *B. pyocyaneus* was growing.

Period of growth	Unsterilised series			Boiled series		
	Streaks of cocci	<i>B. coli</i>	<i>B. pyocyaneus</i>	Cocci	<i>B. coli</i>	<i>B. pyocyaneus</i>
7 days	0	++	0	0	++	++
5 "	*?	++	0	0	++	++
3 "	+	++	*?	+	++	++
2 "	+	++	*?	++	++	++
1 day	+	++	*	++	++	++

0 = no visible colonies. \*? = doubtful growth. \* = very slight growth. + = moderate growth. ++ = abundant growth.

It will be seen that *B. coli* and *B. pyocyaneus* grew well on the medium in which *S. aureus* had been growing, and that on the same medium after boiling a few colonies of the cocci grew.

*S. aureus* grew poorly and *B. pyocyaneus* grew well on the medium in which *B. coli* had been growing, but while *B. coli* itself grew well on this medium after boiling there was little or no growth on the example which had not been boiled.

*S. aureus* grew moderately and *B. coli* grew well on the medium in which *B. pyocyaneus* had been growing, and *B. pyocyaneus* itself grew well on it after it had been boiled.

Taking into consideration the fact that agar seems to inhibit growth to a slight extent and that very small surface colonies are difficult to see, the results of this series of experiments are in general agreement with the results of the more exact series just quoted.

SECTION XI. *The influence of varying the reaction of the medium.*

The experiments hitherto described were carried out in meat extract neutralised to Neutral Red. The experiments quoted in this section, which were amongst the first to be undertaken, were made in order to ascertain the effects of varying the reaction of the medium by the addition of *N*/10 hydrochloric acid or *N*/10 soda on the growth of *Staphylococci* and *B. coli* at 37° C. It will be noticed that the proportion of meat extract differs in these experiments from that used in most of the previous experiments. The addition of certain quantities of both acid and soda caused a precipitate to form. The medium was approximately neutral to neutral red when 0.1 *N*/10 soda was added.

Tube	Meat extract (1 part meat to 4 parts water)	<i>N</i> /10 HCl	Distilled water	Precipitate
1	2.5 c.c.	.5 c.c.	1.3 c.c.	Slight
2	2.5	.4	1.4	Present
3	2.5	.3	1.5	"
4	2.5	.2	1.6	Slight
5	2.5	.1	1.7	None
6	2.5	—	1.8	"
		<i>N</i> /10 soda		
7	2.5	.2	1.6	"
8	2.5	.4	1.4	"
9	2.5	.6	1.2	Slight
10	2.5	.8	1.0	Present
11	2.5	1.0	.8	"
12	2.5	1.2	.6	"
13	2.5	1.6	.2	"
14	2.5	1.8	—	"
15	2.5	2.0	—	"



After inoculation the cultures contained 376 *S. aureus* and 68 *B. coli* per loopful.

Tube	15 hours		48 hours		72 hours	
	Cocci	<i>B. coli</i>	Cocci	<i>B. coli</i>	Cocci	<i>B. coli</i>
1	29	1	226	0	6	0
2	52	0	—	0	1	0
3	304	0	146,170	0	—	5
4	491,200	9,450	6,680,000	71,000	6,824,000	440,000
5	4,616,000	3,264,000	10,560,000	6,688,000	12,040,000	12,248,000
6	7,088,000	5,440,000	14,328,000	13,072,000	15,424,000	14,864,000
7	7,824,000	7,232,000	17,704,000	12,832,000	19,816,000	12,168,000
8	6,928,000	4,272,000	15,990,000	8,304,000	12,288,000	5,600,000
9	2,952,000	3,728	14,496,000	5,592,000	9,176,000	4,968,000
10	58,000	0	10,592,000	0	7,192,000	0
11	1,144	0	9,504,000	0	6,224,000	0
12	130	0	9,408,000	0	7,104,000	0
13	0	0	24	0	5,424,000	0
14	0	0	7	0	1,408,000	0
15	0	0	1	0	0	0

Tube	94 hours		120 hours		168 hours	
	Cocci	<i>B. coli</i>	Cocci	<i>B. coli</i>	Cocci	<i>B. coli</i>
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	296,000	0	4,356,000	0	8,840,000	0
4	8,144,000	316,000	6,504,000	336,000	5,424,000	236,000
5	11,440,000	11,272,000	6,736,000	8,240,000	5,525,000	3,232,000
6	14,736,000	11,424,000	10,860,000	4,704,000	7,752,000	2,712,000
7	11,520,000	8,536,000	8,080,000	4,888,000	4,752,000	2,416,000
8	—	6,072,000	3,424,000	4,024,000	2,944,000	1,688,000
9	6,136,000	4,864,000	4,592,000	4,336,000	1,328,000	2,576,000
10	5,824,000	0	5,224,000	0	2,488,000	0
11	6,240,000	0	4,928,000	0	2,896,000	0
12	5,808,000	0	4,448,000	0	2,424,000	0
13	7,168,000	0	5,344,000	0	2,464,000	0
14	7,352,000	0	5,240,000	0	2,768,000	0
15	0	0	0	0	0	0

Tube	216 hours		16 days		36 days*	
	Cocci	<i>B. coli</i>	Cocci	<i>B. coli</i>	Cocci	<i>B. coli</i>
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	10,360,000	0	864,000	0	392,000	0
4	4,808,000	976,000	408,000	444,000	400,000	192,000
5	1,616,000	2,224,000	496,000	100,000	257,000	116,000
6	4,636,000	1,204,000	544,000	68,000	960,000	106,000
7	2,760,000	2,004,000	404,000	27,500	760,000	12,000
8	1,223,000	1,480,000	228,000	76,000	—	106,000
9	—	—	72,000	—	136,000	—
10	—	0	52,000	0	98,000	0
11	2,324,000	0	144,000	0	—	0
12	1,896,000	0	72,000	0	20,250	0
13	1,668,000	0	96,000	0	6,500	0
14	840,000	0	6,500	0	8,250	0
15	0	0	0	0	0	0

\* Cultures much reduced by evaporation.

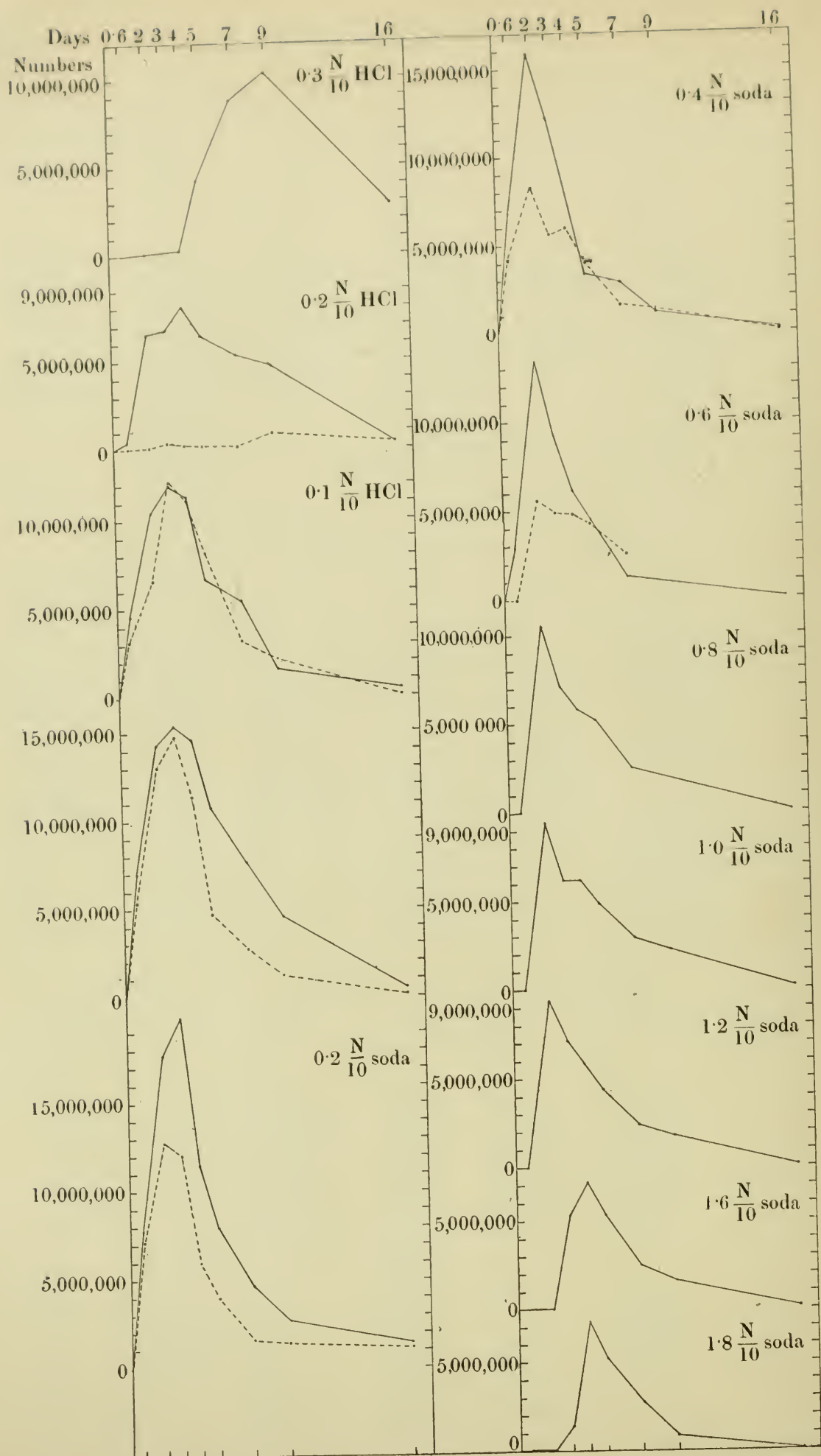


Chart 16. Showing the course of events in cultures of *S. aureus* and *B. coli* at different reactions.



It will be seen that with a small inoculation of cocci when the medium contains .5 c.c. or .4 c.c. *N*/10 hydrochloric acid the organisms decrease in number and are dead on the 4th day. With .3 c.c. *N*/10 hydrochloric acid added to the medium there is at first a slight decrease in the numbers and then a slow rise for four days, followed by a great increase, the numbers reaching a maximum about the 9th day. With 0.2 c.c. *N*/10 hydrochloric acid added there is only a very slight increase in 15 hours, followed by a rapid increase during the next day. The maximum is reached on the 4th day. With 0.1 c.c. *N*/10 hydrochloric acid added the curve resembles that obtained with un-neutralised meat extract, the maximum being reached on the 4th day. The subsequent fall in numbers is moderately rapid.

The effect of adding increasing quantities of *N*/10 hydrochloric acid up to 0.3 c.c. is to retard the growth during the earlier stages of incubation though subsequently rapid growth takes place and a high maximum is reached. Considerable numbers of the cocci survive for a long time. With small primary inoculations the addition of more than 0.3 c.c. *N*/10 hydrochloric acid results in the death of the cocci in a few days.

When 0.2 c.c. *N*/10 soda is added the maximum is reached on the third day and is followed by a rapid fall in numbers. With additions varying between 0.4 and 1.2 c.c. of *N*/10 soda there is a progressive decrease in the height reached by maxima, the rate of growth in the early stages is progressively retarded, and the rate of decrease in numbers seems to be retarded also. With 1.6 c.c. and 1.8 c.c. *N*/10 soda added the rate of growth in the early stages is markedly retarded, and the maxima are not reached till the fourth day. With 2.0 c.c. *N*/10 soda no growth occurs.

In this series of experiments *B. coli* seemed to be more sensitive to the reaction of the medium, especially on the alkaline side, than *S. aureus*, but this may be due partly to the very small number inoculated. In the case of *S. aureus* certain other experiments seem to indicate that the larger the primary inoculation the wider is the range of reaction in which multiplication takes place.

In the case of *B. pyocyaneus* growth does not occur if more than 0.2 c.c. of *N*/10 hydrochloric acid or more than 1.6 c.c. soda are added.

## SECTION XII. *The influence of the addition of small quantities of N/10 hydrochloric acid.*

The two series of experiments here quoted were undertaken to ascertain more precisely than in previous experiments the effects of additions of small quantities of *N*/10 hydrochloric acid. Each tube contained 5 c.c. of *undiluted* meat extract. To tube 1, which acted as a control, no acid was added. To tube 2 0.2 c.c. *N*/10 hydrochloric acid was added and caused the medium to become opalescent; to tube 3 0.3 c.c. *N*/10 hydrochloric acid was added and the fluid became opalescent and a precipitate formed; to tube 4 0.4 c.c.

N/10 hydrochloric acid was added and a considerable precipitate formed; to tube 5 0.7 c.c. N/10 hydrochloric acid was added and a still greater precipitate formed. In both series of experiments a drop of an emulsion of *S. aureus* was added to each tube, but while in the first series the medium

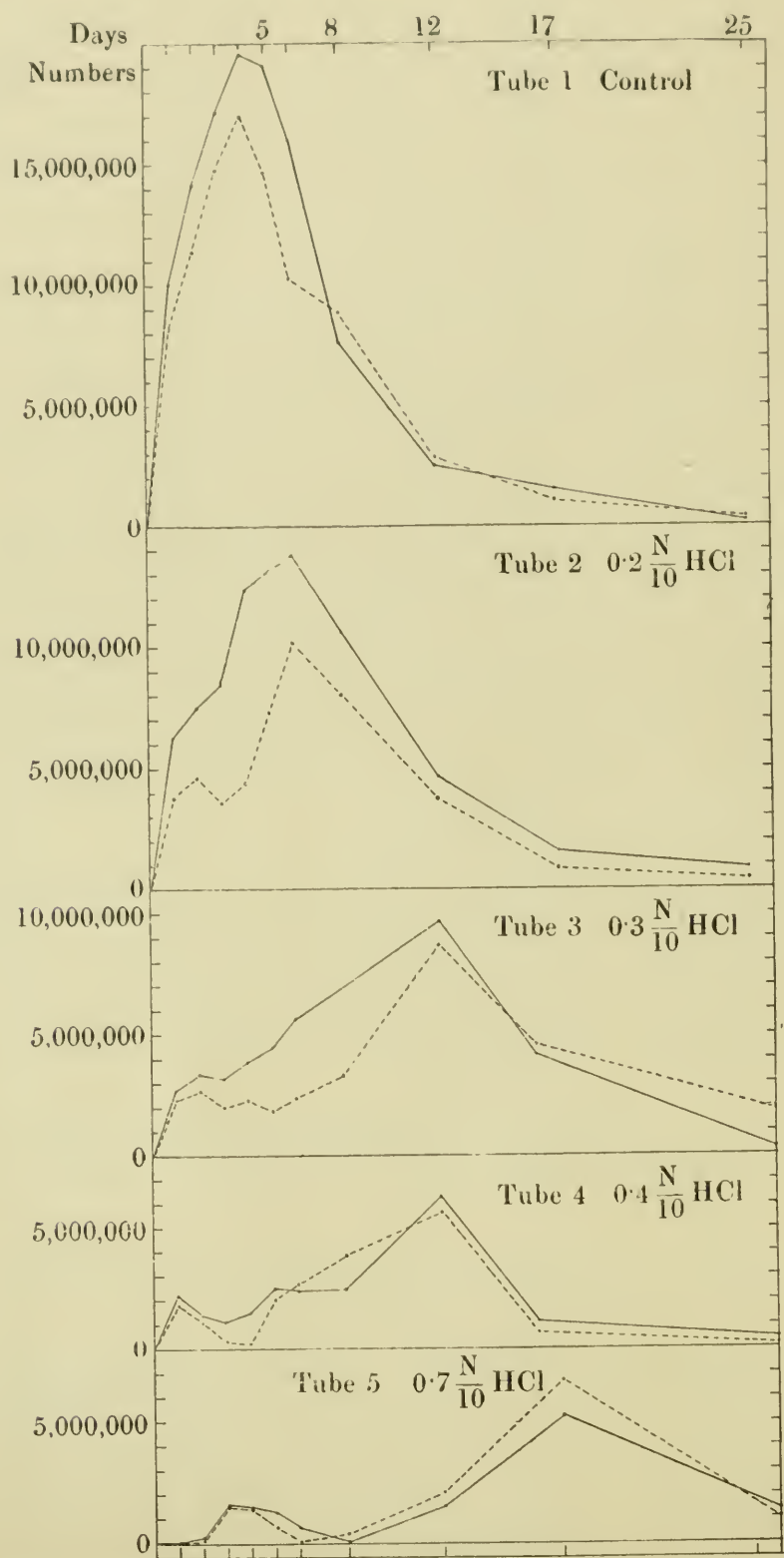


Chart 17. Showing the influence of the addition of various quantities of N/10 HCl to meat extract cultures. Series I=continuous line, Series II=broken line.



after inoculation contained 168 organisms per loop, in the second series it contained only 13 per loop.

		5 hours		1st day		2nd day	
Tube		Series I	Series II	Series I	Series II	Series I	Series II
1	—	3200	190	10,112,000	8,192,000	14,168,000	11,432,000
2	0.2 c.c. <i>N</i> /10 HCl	1968	88	6,368,000	3,856,000	7,584,000	4,616,000
3	0.3 „	688	37	2,784,000	2,392,000	3,432,000	2,780,000
4	0.4 „	244	28	2,216,000	1,808,000	1,432,000	1,112,000
5	0.7 „	33	6	298	4	228,000	100,000

		3rd day		4th day		5th day	
Tube		Series I	Series II	Series I	Series II	Series I	Series II
1	—	17,256,000	14,872,000	19,609,000	17,088,000	19,120,000	14,752,000
2	0.2 c.c. <i>N</i> /10 HCl	8,496,000	3,640,000	12,416,000	4,456,000	13,209,000	7,368,000
3	0.3 „	3,192,000	2,048,000	3,944,000	2,304,000	4,472,000	1,920,000
4	0.4 „	1,136,000	362,000	1,544,000	203,000	2,560,000	2,096,000
5	0.7 „	1,640,000	1,608,000	1,556,000	1,516,000	1,372,000	676,000

		6th day		8th day		12th day	
Tube		Series I	Series II	Series I	Series II	Series I	Series II
1	—	16,016,000	10,320,000	7,648,000	8,968,000	2,556,000	2,908,000
2	0.2 c.c. <i>N</i> /10 HCl	13,824,000	10,200,000	10,648,000	8,064,000	4,732,000	3,808,000
3	0.3 „	5,768,000	2,472,000	—	3,336,000	9,740,000	8,704,000
4	0.4 „	2,392,000	2,632,000	2,440,000	3,896,000	6,336,000	5,768,000
5	0.7 „	716,000	75,000	25,000	392,000	1,515,000	2,118,000

		17th day		25th day	
Tube		Series I	Series II	Series I	Series II
1	—	1,560,000	1,020,000	284,000	300,000
2	0.2 c.c. <i>N</i> /10 HCl	1,664,000	909,000	992,000	442,000
3	0.3 „	4,175,000	4,624,000	367,900	1,828,000
4	0.4 „	1,144,000	702,000	494,000	186,000
5	0.7 „	5,200,000	6,768,000	1,490,000	918,000

In passing down the series it will be seen that the type of curve gradually changes from a "standard" with one peak to a curve with two peaks, separated by an interval in which the numbers are small. The second series containing the smaller initial dose seems to be the most influenced.

### SECTION XIII. *The effects of cultivating organisms for prolonged periods on media of different reactions.*

Three series of tubes containing media of the following compositions were prepared:

Series	Meat extract	Distilled water	
I	2.5 c.c.	1.5 c.c.	0.3 c.c. <i>N</i> /10 HCl
II	2.5	1.8	—
III	2.5	0	1.8 c.c. <i>N</i> /10 NaOH

Series I and III correspond to tubes 3 and 14 of Section XI, the most acid and most alkaline of that series in which satisfactory growth took place. A tube of each series was inoculated with an emulsion of the *S. aureus*, and

incubated at 37° C. and subcultures into similar tubes were made weekly for ten weeks. Then subcultures from each were grown for 24 hours at 37° C. on solid media made by adding agar to fluids of the composition mentioned. Consequently the cocci in Series I had been growing on an acid medium, those of Series II on a nearly neutral medium and those of Series III on an alkaline medium for ten weeks. They are referred to subsequently as acid, neutral and alkaline acclimatised cocci respectively. In order to ascertain the effects of this acclimatisation tubes of the same composition as Series I, II, and III were inoculated with emulsions of the cocci from each of the agar cultures. The whole series of experiments therefore included nine cultures, namely :

						Immediate count of numbers
Acid acclimatised cocci inoculated into acid meat extract, A I						82
neutral ,, ,, A II						—
alkaline,, ,, A III						—
Neutral	,,	,,	acid ,, ,, B I			—
			neutral ,, ,, B II			992
			alkaline,, ,, B III			—
Alkaline	,,	,,	acid ,, ,, C I			—
			neutral ,, ,, C II			—
			alkaline,, ,, C III			360
Tube	1 day	2 days	3 days	4 days	5 days	6 days
A I	122,500	2,928,000	2,560,000	2,640,000	4,088,000	6,544,000
II	5,280,000	10,192,000	10,256,000	9,344,000	9,760,000	8,432,000
III	2,256,000	2,248,000	1,528,000	680,000	276,000	220,000
B I	4,830	1,240,000	1,760,000	2,416,000	2,376,000	1,680,000
II	7,616,000	12,080,000	11,184,000	11,088,000	11,520,000	11,966,000
III	5,616,000	2,784,000	2,156,000	1,264,000	554,000	472,000
C I	65	544,000	1,752,000	2,176,000	2,584,000	744,000
II	9,840,000	12,688,000	13,664,000	14,176,000	13,844,000	14,480,000
III	7,240,000	5,168,000	3,336,000	1,776,000	980,000	864,000
7 days 8 days 9 days 10 days 11 days 12 days						
A I	10,752 000	8,768,000	6,616,000	5,456,000	4,464,000	2,056,000
II	4,460,000	1,056,000	1,840,000	1,560,000	1,456,000	488,000
III	240,000	328,000	252,000	336,000	312,000	220,000
B I	944,000	560,000	732,000	3,820,000	5,584,000	9,280,000
II	9,936,000	7,856,000	4,592,000	2,576,000	1,960,000	1,104,000
III	220,000	204,000	132,000	80,000	46,500	10,500
C I	556,000	340,000	252,000	264,000	1,184,000	7,071,000
II	13,456,000	8,720,000	2,680,000	1,184,000	960,000	2,000,000
III	560,000	492,000	196,000	70,500	53,000	10,000
13 days 14 days 15 days 16 days 17 days 18 days						
A I	1,992,000	2,384,000	220,000	160,000	324,000	404,000
II	256,000	180,000	380,000	784,000	996,000	1,088,000
III	148,000	56,000	40,500	26,000	6,000	4,500
B I	9,680,000	8,768,000	7,376,000	5,728,000	4,336,000	208,000
II	380,000	220,000	3,248,000	5,632,000	6,544,000	6,240,000
III	12,500	9,500	13,500	17,000	18,000	8,000
C I	9,880,000	11,250,000	11,936,000	10,456,000	8,768,000	4,304,000
II	1,832,000	1,784,000	1,528,000	1,200,000	704,000	296,000
III	107,000	96,000	66,000	24,000	5,500	500



Tube	19 days	20 days	21 days	22 days	23 days	24 days *
A I	448,000	268,000	280,000	292,000	292,000	3,424,000
II	852,000	564,000	308,000	556,000	780,000	2,252,000
III	13,500	17,350	5,888	2,720	2,336	1,896,000
B I	168,000	93,000	148,000	116,000	80,000	4,944,000
II	768,000	161,000	36,000	—	25,500	2,400,000
III	1,500	172	5	9	1	1,645,000
C I	2,936,000	1,148,000	592,000	176,000	124,000	4,416,000
II	132,000	152,000	180,000	232,000	184,000	4,206,000
III	—	544	2,384	2,816	3,568	3,384,000
	25 days	26 days	27 days	28 days	29 days	30 days
A I	4,736,000	5,264,000	4,096,000	3,680,000	2,216,000	784,000
II	2,144,000	1,384,000	904,000	1,216,000	998,000	880,000
III	2,184,000	1,704,000	1,360,000	198,000	20,500	9,500
B I	6,320,000	5,096,000	3,944,000	1,816,000	800,000	520,000
II	3,632,000	5,256,000	4,328,000	2,125,000	576,000	170,000
III	2,720,000	1,756,000	1,328,000	668,000	100,000	33,500
C I	5,200,000	4,576,000	1,968,000	912,000	348,000	204,000
II	4,720,000	4,136,000	1,856,000	772,000	418,000	304,000
III	2,224,000	1,840,000	1,268,000	1,080,000	616,000	248,000

\* After the samples for counting had been removed, the contents of the tubes were made up to 5 c.c. with sterile distilled water, and one drop of a concentrated meat extract (50 c.c. concentrated to 2 c.c. by evaporation at 40° C.) added.

It will be noticed that in the case of the *acid acclimatised coccus* in the acid medium (A I) moderate growth occurred in 24 hours and considerable multiplication in two days. During the next two days no further multiplication took place, but subsequently rapid multiplication occurred, the maximum numbers being reached on the 7th day. After this there was a rapid decline. In the neutral medium (A II) there was rapid multiplication during the first two days. During the next four days a high level was maintained followed by a rapid fall. A small secondary rise commenced on the fifteenth day. In the alkaline medium (A III) moderate multiplication occurred in the first 24 hours followed by a slow decline to a very low level.

In the case of the *neutral acclimatised coccus* in the acid medium (B I) very little multiplication occurred in the first 24 hours. During the next three days slow multiplication took place followed by a slow fall in numbers during the next five days. After this rapid multiplication occurred the maximum being reached on the 13th day. A rapid fall in numbers followed. In the neutral medium (B II) a high level was reached on the second day, and maintained for four days. After a rapid fall in numbers a marked secondary rise occurred, commencing on the 14th day. In the alkaline medium (B III) rapid multiplication occurred in the first 24 hours followed by a fall in the numbers to a low level.

In the case of the *alkali acclimatised coccus* in the acid medium (C I) after a moderate primary multiplication which reached its maximum on the 5th day, a fall in numbers occurred followed by a rapid and great multiplica-

tion reaching its maximum on the 15th day. In the neutral medium (C II) a high level was reached on the second day, and maintained for five days. This was followed by a rapid fall and a small secondary rise commencing on

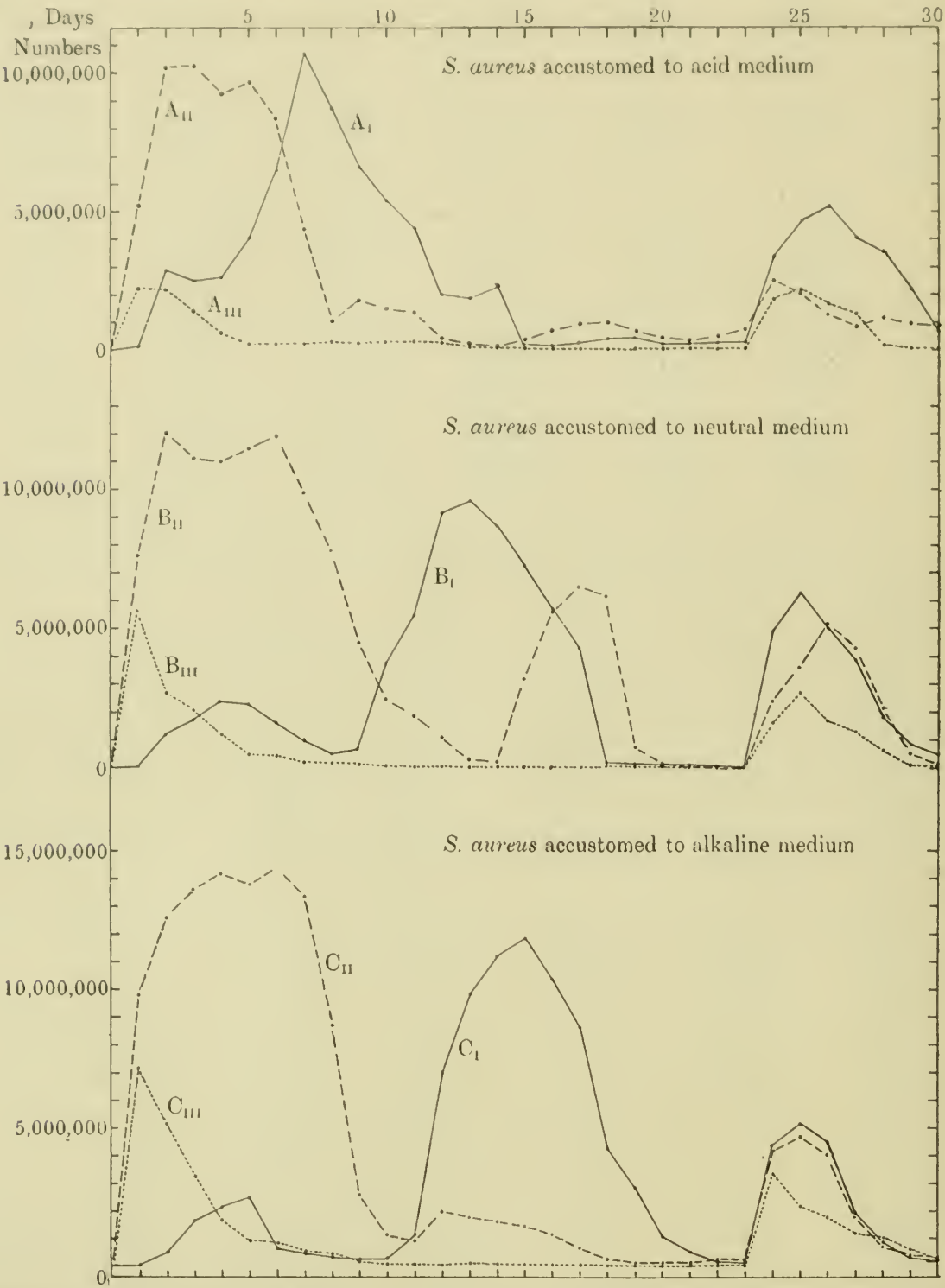


Chart 18. Showing the rate of multiplication of acid, neutral and alkali acclimatised cocci in acid, neutral and alkaline media respectively.

the 12th day. In the alkaline medium (C III) rapid multiplication occurred in the first 24 hours followed by a somewhat rapid fall to a low level.

The addition of further food material on the 23rd day caused considerable



multiplication in all the cultures, which was most marked in the acid media (*A I*, *B I*, *C I*).

The influence of the previous treatment is perhaps best seen in Chart 19, in which the growths of the three treated strains are compared on similar media.



Chart 19. Comparing the rate of multiplication on acid, neutral and alkaline meat extract respectively of acid, neutral and alkali acclimatised cocci.

It will be seen that on the acid medium all three strains show a small primary rise followed after a fall in numbers by a great secondary rise. In the case of the acid acclimatised coccus the secondary rise reaches its maximum on the 7th day, in the neutral acclimatised coccus on the 13th day and in

the alkali acclimatised coccus on the 15th day. In the neutral medium there is also a primary and a secondary rise, but the former is much greater than the latter. In the case of the acid acclimatised coccus the primary rise is least in height and duration and in the alkali acclimatised coccus greatest both in height and duration. In the alkaline medium a primary rise only occurs, and subsequently the numbers fall to a very low level. The rise is least in the acid acclimatised coccus, and greatest in the alkali acclimatised coccus.

In comparing these experiments with others previously quoted it should be remembered that the organisms have not only been acclimatised to growth in media of different reactions, but also to continuous growth in fluid meat extract medium.

#### SECTION XIV. *The influence on growth of the addition of gelatin or agar to meat extract.*

In order to ascertain the influence of the addition of small quantities of gelatin or agar to meat extract five series of tubes containing media of the following composition were prepared and sterilised in the autoclave.

Series	Meat extract	N/10 soda	Gelatin (20 %) in distilled water	Agar (2 %) in distilled water	Distilled water
<i>A</i>	1	0.26	2	—	1.74
<i>B</i>	0	0.18	2	—	2.82
<i>C</i>	1	0.08	—	—	3.92
<i>D</i>	1	0.08	—	0.5	3.42
<i>E</i>	0	0	—	0.5	4.5

All the series were made neutral to neutral red by the addition of *N/10* soda. It will be noticed that Series *A* contains both meat extract and gelatin, Series *B* gelatin alone, Series *C* acts as a control, Series *D* contains meat extract and agar, and Series *E* agar alone.

Four tubes of each series were prepared and numbered *A* 1, 2, 3, 4, etc. To the first tube of each series a drop of a strong emulsion of *S. aureus* was added; the 2nd, 3rd and 4th tubes received such dilutions of the emulsion that approximately  $\frac{1}{100}$ ,  $\frac{1}{10,000}$ , and  $\frac{1}{100,000}$  of the dose was added.

In order to avoid confusion three experiments only of each series are illustrated in Chart 20. It will be noticed that the appearances in the charts are affected in each series only to a very slight degree by variations in the initial dose of the organisms. Series *C*, the controls, show "standard" curves. In Series *B*, containing gelatin only, multiplication occurs, but only to about one-fifth the extent in Series *C*. On the other hand in Series *A*, containing both meat extract and gelatin, multiplication occurs to twice the extent it does in Series *C*, the maximum figures being greater by one-third than the maxima of *B* and *C* together. In Series *D*, agar and meat extract, multiplication occurs to only half the extent it does in Series *C*, but the fall in the



		Immediately after inoculation	6.5 hours	24 hours	30 hours	54 hours	72 hours	96 hours		
	<i>A</i>	1	—	2,856,000	8,658,000	11,280,000	14,096,000	15,808,000	16,464,000	
		2	—	194,000	6,320,000	9,472,000	13,288,000	15,000,000	16,484,000	
		3	—	1,520	—	—	—	—	—	
		4	—	21	3,952,000	5,988,000	10,848,000	14,864,000	15,502,000	
	<i>B</i>	1	—	1,116,000	1,752,000	2,096,000	1,568,000	1,320,000	288,000	
		2	—	41,600	1,664,000	1,720,000	1,856,000	1,656,000	432,000	
		3	—	328	1,368,000	1,798,000	2,096,000	2,004,000	332,000	
		4	—	3	931,000	1,656,000	2,120,000	1,952,000	336,000	
	<i>C</i>	1	78,048	2,472,000	6,620,000	7,744,000	9,200,000	9,568,000	7,424,000	
		2	872	57,600	5,744,000	7,376,000	8,604,000	9,344,000	8,260,000	
		3	7	248	5,166,000	6,522,000	9,088,000	9,088,000	7,232,000	
		4	0.8	1	2,800,000	5,984,000	8,500,000	—	6,670,000	
	<i>D</i>	1	—	1,832,000	4,848,000	5,100,000	5,232,000	3,568,000	—	
		2	—	44,000	3,520,000	4,944,000	4,932,000	3,776,000	—	
		3	—	?	2,464,000	4,624,000	5,166,000	3,904,000	—	
		4	—	6	1,528,000	4,688,000	4,848,000	4,688,000	—	
	<i>E</i>	1	—	75,136	2,850	—	—	0	0	
		2	—	145	0	—	—	0	0	
		3	—	7	0	—	—	0	0	
		4	—	0	0	—	—	0	0	
Tube	5 days	6 days	7 days	8 days	9 days	10 days	11 days	13 days	14 days	
<i>A</i>	1	17,120,000	17,096,000	10,496,000	7,792,000	5,924,000	5,166,000	4,208,000	244,000	232,000
	2	17,280,000	17,344,000	8,672,000	7,968,000	6,152,000	5,832,000	4,240,000	592,000	232,000
	3	—	—	—	—	—	—	—	—	—
	4	16,716,000	16,604,000	13,288,000	11,536,000	9,296,000	8,528,000	4,320,000	744,000	520,000
<i>B</i>	1	196,000	228,000	188,000	228,000	148,000	—	—	—	82,000
	2	240,000	212,000	192,000	148,000	228,000	—	—	—	44,500
	3	320,000	172,000	164,000	172,000	204,000	—	—	—	43,000
	4	276,000	184,000	184,000	250,000	224,000	—	—	—	66,000
<i>C</i>	1	3,856,000	704,000	324,000	160,000	224,000	—	—	—	83,000
	2	5,936,000	1,080,000	308,000	272,000	232,000	—	—	—	42,000
	3	—	—	260,000	152,000	144,000	—	—	—	145,000
	4	5,120,000	1,664,000	460,000	244,000	114,000	—	—	—	67,000
<i>D</i>	1	2,368,000	1,456,000	1,628,000	1,136,000	1,144,000	736,000	284,000	29,500	—
	2	2,920,000	2,200,000	1,648,000	1,520,000	1,588,000	1,312,000	932,000	136,000	—
	3	2,512,000	1,944,000	1,632,000	1,600,000	1,696,000	1,440,000	792,000	104,000	—
	4	3,120,000	2,304,000	1,416,000	1,326,000	1,392,000	1,112,000	420,000	46,000	—

numbers is much more prolonged. In Series *E*, agar alone, no multiplication took place, and the cocci died out rapidly.

From experiments of this nature, of which three were carried out, it is evident that *S. aureus* can grow in gelatin alone, and that the addition of gelatin to meat extract results in a medium, which is very favourable to multiplication. *S. aureus* cannot grow on agar alone, and the addition of agar to meat extract checks multiplication, but causes the decline in numbers to be slower.

Organisms belonging to other groups may react differently under such experimental conditions.

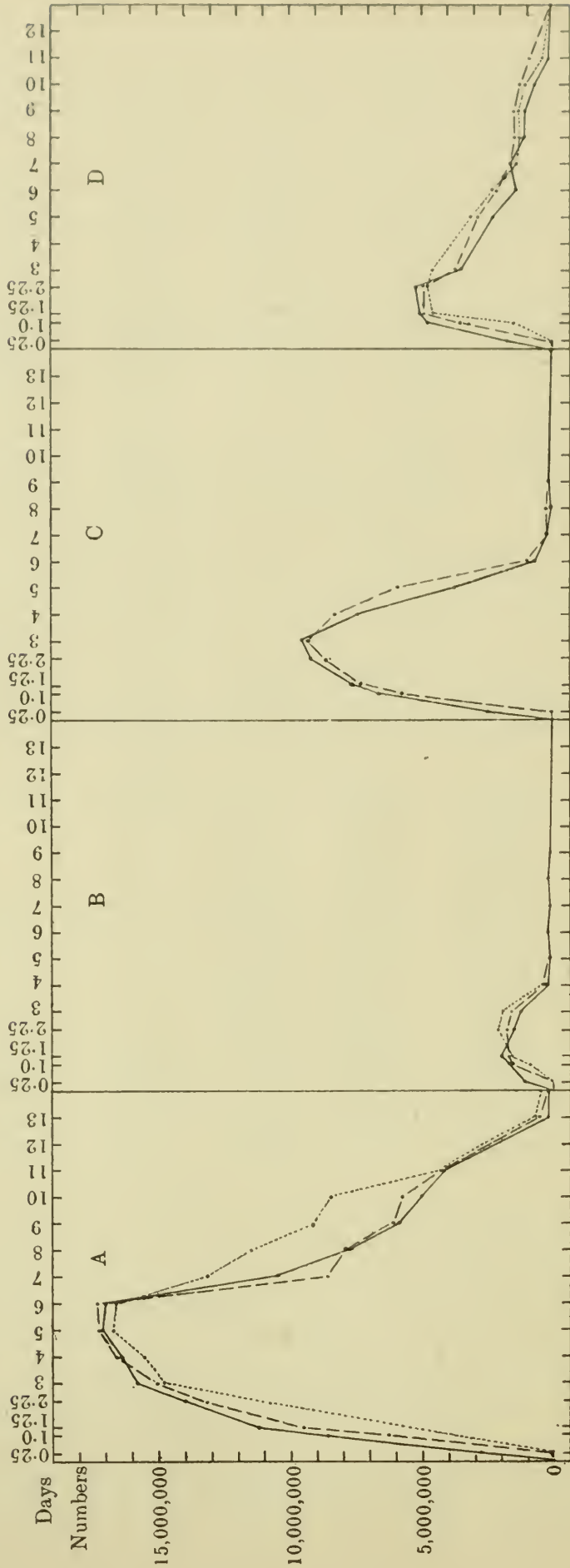


Chart 20. Showing the rate of multiplication of *S. aureus* in A, gelatin and meat extract; B, gelatin only; C, meat extract only; and D, agar and meat extract.



SECTION XV. *The effects of adding various quantities of different acids to neutral meat extract agar before inoculation.*

The experiments described in this section were carried out only once, and not repeated. They are described here as they are related to those recounted in the last section and exhibit some interesting features.

The medium, consisting of meat extract (1 vol. of meat extract to 2.5 vols. of water) and agar (2 per cent.), was cleared with egg-white, neutralised to neutral red and sterilised on three occasions in the steamer. While hot 9 c.c. portions were pipetted into large test-tubes. To a second series sufficient salt was added to make a concentration of 0.9 per cent. Neither series contained peptone.

At the commencement of the experiment the agar in the tubes was melted and cooled to 45° C. To different tubes 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 c.c. of *N/10* solutions of the following acids<sup>1</sup>, hydrochloric, orthophosphoric, lactic, butyric, isobutyric and a mixture of glutaminic and aspartic, were added, and the contents of the tubes mixed by rotation. Then one drop of an emulsion of *S. aureus* in distilled water was added, the presence or absence of a precipitate noted, the tubes rotated and plates poured. After 24 hours' incubation at 37° C. the colonies present on each plate were counted.

Quantity of acid added	<i>N/10</i> hydrochloric acid			
	Medium without salt		0.9 % salt added	
	Colonies	Condition of medium	Colonies	Condition of medium
0.25 c.c.	1946	Clear	1018	Clear
0.5	1720	„	1725	Slight opalescence
0.75	1990	„	1550	Clear
1.0	0	Marked precipitate	1116	„
1.5	0	„ „	1890	„
2.0	1988	Clear	1527	„
3.0	0	Marked precipitate	1076	Slight precipitate
	<i>N/10</i> orthophosphoric acid			
	Colonies	Condition of medium	Colonies	Condition of medium
0.25	1704	Slight opalescence	491	Very slight opalescence?
0.5	1995	Clear	1960	„ „
0.75	1775	„	1297	„ „
1.0	1918	„	928	Clear
2.0	1	Precipitate	0	Precipitate
3.0	1521	Slight opalescence	664	Clear
4.0	0	Precipitate	1675	„
5.0	0	„	0	Precipitate
	<i>N/10</i> lactic acid			
	Colonies	Condition of medium	Colonies	Condition of medium
0.25	1908	Clear	1564	Clear
0.5	1843	„	1586	Slight opalescence
0.75	1786	Slight precipitate	1313	„ „
1.0	1835	Clear	1491	„ „
2.0	1889	„	0	Precipitate
3.0	0	Precipitate	0	„
4.0	0	„	0	„
5.0	0	„	0	„

<sup>1</sup> These solutions were made up by Mr F. W. Foreman.

*Bacteria in Fluid Cultures*

Quantity of acid added	<i>N</i> /10 butyric acid			
	Medium without salt		0.9 % salt added	
	Colonies	Condition of medium	Colonies	Condition of medium
0.25	1939	Clear	1383	Clear
0.5	1663	"	916	"
0.75	794	Slight opalescence	1863	Slight opalescence
1.0	1545	Clear	252	Precipitate
1.5	0	Precipitate	1	"
2.0	—	—	0	"
	<i>N</i> /10 isobutyric acid			
	Colonies	Condition of medium	Colonies	Condition of medium
	Colonies	Condition of medium	Colonies	Condition of medium
0.25	1846	Slight opalescence	1692	Very slight opalescence
0.5	1989	" "	1260	" "
0.75	1831	Clear	1753	" "
1.0	0	Precipitate	0	Precipitate
1.5	1868	Clear	0	"
2.0	0	Precipitate	0	"
	<i>N</i> /10 glutaminic and aspartic acids (calculated as if all glutaminic)			
	Colonies	Condition of medium	Colonies	Condition of medium
	Colonies	Condition of medium	Colonies	Condition of medium
0.25	2134	Very slight opalescence	1826	Clear
0.5	1847	Slight precipitate	1867	Slight opalescence
0.75	1804	" "	1708	" precipitate
1.0	1282	Precipitate	1817	Clear
2.0	0	"	0	Precipitate
3.0	0	"	1778	Clear
4.0	0	"	0	Precipitate
5.0	0	"	0	"

In the series without salt it will be noticed that in the case of hydrochloric, orthophosphoric and isobutyric acids growth occurred when the medium remained clear after the addition of the acid, but not when the addition of the acid caused a precipitate. In the case of butyric acid the addition of 0.75 c.c. caused a slight opalescence, and the number of colonies was greatly reduced, but in the case of lactic acid though the addition of 0.75 c.c. caused a precipitate little reduction in the number of colonies occurred. In the case of glutaminic acid growth occurred when quantities up to 1.0 c.c. were added in spite of the production of a precipitate.

In the series containing 0.9 per cent. of salt most of the tubes to which hydrochloric acid was added remained clear and growth occurred in all of them. In the case of orthophosphoric acid a well marked precipitate formed on the addition of 2.0 c.c. and at that point no growth occurred. In the tubes to which 1.0 and 3.0 c.c. were added the number of colonies was small. In the case of lactic, butyric and isobutyric acids inhibition of growth was caused by the addition of smaller quantities than in the series to which no salt was added. In the case of glutaminic acid a precipitate was formed on the addition of 0.75 and 2.0 c.c. At the latter point only was growth inhibited.



SECTION XVI. *The effects of the addition of glucose.*

Several series of experiments were undertaken to ascertain the effects of the addition of glucose to meat extract media. The results were not uniform, and therefore the experiments are quoted in the order in which they were carried out.

Series I. Four tubes containing meat extract 1 c.c., *N*/10 soda 0.08 c.c., glucose (5 per cent.) in distilled water 1 c.c. and distilled water 2.92 c.c. were prepared, and each was inoculated with a drop of an emulsion of *S. aureus* in distilled water and incubated at 37° C.

Tubo	Number at beginning of experiment	1 day	2 days	3 days	4 days	5 days
1	4416	4,000,000	3,288,000	1,120,000	116,000	2,500
2	—	3,888,000	3,776,000	1,744,000	316,000	7,000
3	—	3,632,000	3,648,000	1,712,000	352,000	12,000
4	—	4,060,000	3,376,000	1,536,000	160,000	4,000
	6 days	7 days	8 days	12 days	13 days	14 days
1	62	3	1	0	0	0
2	74	1	0	0	0	0
3	22	0	0	0	0	0
4	73	1	0	0	0	0

All the members of this series behaved in a very uniform manner (Chart 21), and all the cultures were dead by about the 9th day. These cultures differed in appearance from those to which glucose was not added. After 24 hours' incubation there was a copious, granular deposit in the glucose cultures, whereas in those without glucose the fluid was cloudy, and the sediment smaller in quantity and finely divided. Microscopically the organisms in the former were in groups, while in the latter they were separate.

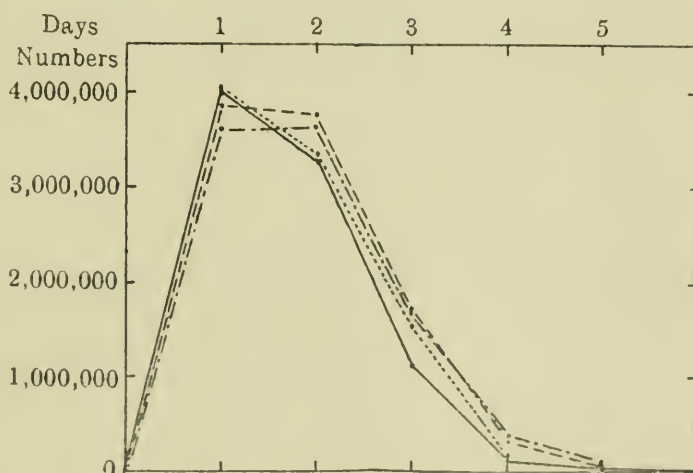


Chart 21. Showing the rate of multiplication in meat extract to which 1 % glucose was added.

In Series II the first three tubes contained different proportions of glucose, the fourth tube contained no glucose and acted as a control, and the last three tubes were of the same composition as tube 3, but additions were made to them daily. To tube 5 was added daily one drop of mixture *A*, to tube 6 one drop of mixture *B* and to tube 7 one drop of mixture *C*.

In making up mixtures *A* and *B* concentrated meat extract obtained by evaporating 40 c.c. of meat extract to 2 c.c. was made use of.

Mixture *A* consisted of 1 c.c. concentrated meat extract and 1 c.c. distilled water

„ *B* „ 1 c.c. „ „ and 1 c.c. glucose (5 %) in distilled water  
 „ *C* „ 1 c.c. distilled water and 1 c.c. „ „ „

Tube	Meat extract	Distilled water	N/10 soda	Glucose (5 %) in distilled water	
1	1 c.c.	0.92	0.08	3.0	
2	1	1.92	0.08	2.0	
3	1	2.92	0.08	1.0	
4	1	3.92	0.08	0	
5	1	2.92	0.08	1.0	1 drop of mixture <i>A</i> added daily
6	1	2.92	0.08	1.0	„ „ <i>B</i> „ „
7	1	2.92	0.08	1.0	„ „ <i>C</i> „ „

Each tube was inoculated with one drop of an emulsion of *S. aureus* in distilled water, and incubated at 37° C.

Tube	No. at beginning of experi- ment	21 hours	45 hours	3 days	4 days	5 days	6 days	7 days
1	5660	4,816,000	2,168,000	352,000	101,000	70,270	98,432	440,000
2	—	5,024,000	2,552,000	696,000	48,000	8,080	1,784	7,392
3	—	5,152,000	3,264,000	1,152,000	83,000	4,368	3,000	808
4	—	7,680,000	9,872,000	5,840,000	4,016,000	2,560,000	1,376,000	732,000
5	—	4,720,000	3,686,000	1,132,000	234,000	69,000	644,000	2,040,000
6	—	4,962,000	4,296,000	1,848,000	321,000	69,000	42,000	179,700
7	—	4,928,000	3,600,000	1,748,000	180,000	40,000	7,500	203
	8 days	9 days	10 days	11 days	12 days	13 days	14 days	15 days
1	234,000	14,500	1,000	2,040	6,240	8,836	7,232	2,544
2	52,700	240,000	268,000	200,000	27,500	1,032	—	79
3	175	5	1	0	0	0	0	0
4	492,000	148,000	48,000	27,500	9,984	9,696	12,736	39,040
5	1,256,000	988,000	76,000	228,000	556,000	496,000	340,000	248,000
6	1,016,000	816,000	124,000	348,000	504,000	684,000	1,056,000	1,144,000
7	42	118	1,064	31,250	64,000	71,500	28,000	3,984
	16 days	17 days	18 days	19 days	20 days	21 days	22 days	23 days
1	1,904	4,544	8,960	2,000	2,064	1,728	5,776	10,880
2	81	66	82	96	90	81	74	49
3	0	0	0	0	0	0	0	0
4	—	—	—	—	—	—	—	—
5	508,000	856,000	1,176,000	268,000	244,000	224,000	145,000	82,000
6	628,000	808,000	664,000	596,000	604,000	716,000	568,000	620,000
7	960	138	9	11	12	33	354	1,496
	24 days	25 days	26 days	27 days	28 days	29 days	30 days	31 days
1	10,596	11,712	14,688	15,488	14,816	16,566	8,400	32,040
2	28	18	17	20	23	35	34	48
3	0	0	0	0	0	0	0	0
4	—	—	—	—	—	—	—	—
5	66,500	51,500	72,000	102,000	166,000	198,000	350,000	548,000*
6	992,000	760,000	708,000	712,000	448,000	348,000	429,000	500,000†
7	4,704	10,272	19,648	21,344	16,128	7,728	488	22

\* 9 drops of mixture *A* added.

† 10 drops of mixture *B* added. After the 32nd and 31st days respectively no additions were made to tubes 5 or 6.



	32 days	33 days	34 days	35 days	36 days	37 days	38 days	39 days
1	15,488	3,686	480	53	20	2	0	0
2	38	31	9	1	1	0	0	0
3	0	0	0	0	0	0	0	0
4	—	—	—	—	—	—	—	—
5	1,032,000 ‡	1,648,000	2,816,000	2,856,000	3,176,000	3,003,000	2,216,000	1,144,000
6	796,000	2,024,000	2,650,000	3,040,000	3,224,000	2,968,000	3,392,000	1,672,000
7	1	0	0	0	0	0	0	0
	40 days	41 days	43 days	44 days	47 days			
1	0	—	0	0	0			
2	0	—	0	0	0			
3	0	—	0	0	0			
4	—	—	—	—	—			
5	196,000	13,500	0	0	0			
6	308,000	2,500	0	0	0			
7	0	—	0	0	0			

‡ 10 drops of mixture A added.

From the tables it will be seen that culture 3 behaved in the same manner as the cultures of Series I. On the other hand culture 1 containing three times as much glucose showed several oscillations in numbers, and small numbers of organisms were alive up to the 37th day. The same phenomenon was exhibited in culture 2, but to a lesser extent. In all three cultures death of the organisms ultimately occurred. In culture 5 to which concentrated meat extract was added daily considerable oscillations occurred, but the figures remained throughout at a moderately high level. The addition of larger quantities of concentrated meat extract on the 31st and 32nd days caused a decided rise in the numbers. Subsequently no additions were made and the culture died. Culture 6 to which concentrated meat extract together with glucose was added daily followed a very similar course to culture 5. These two cultures seem to show that if in the presence of glucose small quantities of food material are added daily, whether with or without glucose, the cultures remain alive for prolonged periods. The addition of larger quantities of food material causes considerable multiplication to take place, but in the absence of further additions of food material the organisms die. With daily small additions of glucose as in culture 7 the organisms may remain alive for 30 days and small oscillations in the numbers occur (Chart 22).

Series III. In this series the proportion of glucose varied in all the tubes, which contained media of the following compositions:

Tube	Meat extract	N/10 soda	Glucose (25 %)	
			Distilled water	in distilled water
1	1 c.c.	0·08	1·92	2
2	1	0·08	2·92	1
3	1	0·08	3·42	0·5
4	1	0·08	3·72	0·2
5	1	0·08	3·82	0·1
6	1	0·08	3·9	0·02
7	1	0·08	3·92	0

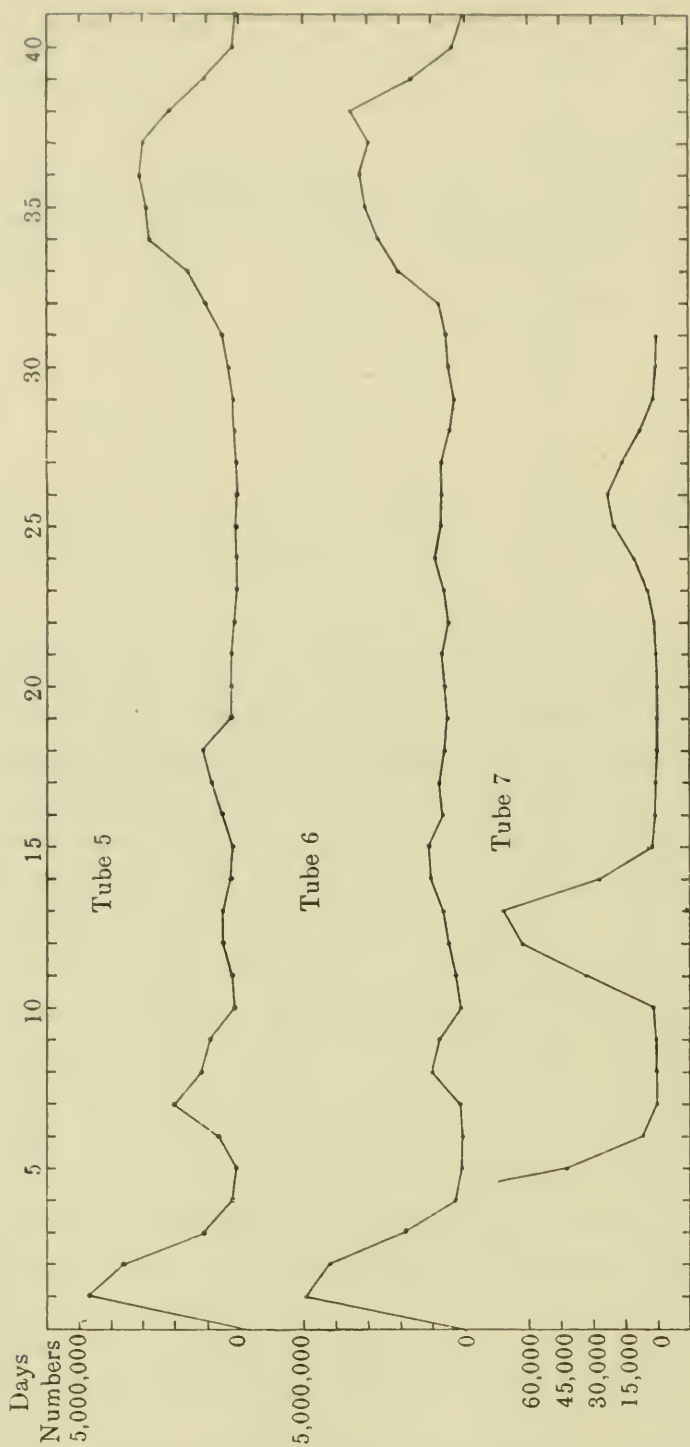


Chart 22. Showing multiplication in tubes of meat extract containing 1 % glucose. Up to the 30th day daily additions were made of meat extract to tube 5, meat extract and glucose to tube 6, and glucose to tube 7. On the 31st and 32nd days large additions were made to tube 5, and on the 31st day to tube 6. Subsequently no additions were made. Tube 7 is illustrated on a larger scale in order to show more clearly the remarkable late fluctuations.

Each tube was inoculated with a drop of an emulsion of *S. aureus* in distilled water and incubated at 37° C. The organisms had been subcultured daily on agar for four days.

Tube	No. at beginning of experiment	23 hours	47 hours	67 hours	4 days	5 days
1	5760	5,616,000	1,260,000	600,000	46,500	8,400
2	—	6,160,000	1,414,000	128,000	10,000	824
3	—	7,137,000	3,052,000	298,000	12,000	1,264
4	—	7,796,000	4,904,000	1,192,000	8,000	208
5	—	8,656,000	5,592,000	2,408,000	660,000	1,906
6	—	8,832,000	7,424,000	3,536,000	1,720,000	296,000
7	—	9,696,000	12,976,000	11,248,000	2,864,000	868,000



Tube	6 days	7 days	8 days	9 days	10 days	11 days
1	2,708	228	36	1	0	0
2	63	0	0	0	0	0
3	190	0	0	0	0	0
4	22	0	0	0	0	0
5	77	8	—	—	384	0
6	55,808	26,176	26,200	26,288	7,536	3,760
7	1,244,000	408,000	272,000	148,000	79,500	46,500
	12 days	13 days	14 days	17 days	22 days	
1	0	—	—	0	0	
2	0	—	—	0	0	
3	0	—	—	0	0	
4	0	—	—	0	0	
5	0	—	—	0	0	
6	856	1,248	888	568	14	
7	13,824	29,056	30,400	45,440	48,128	

Even when the quantity of glucose present is very small the numbers begin to fall after 24 hours' incubation, instead of rising as they do in cultures without glucose (Chart 23).

With increasing quantities of glucose the maximum attained diminishes, and the rate of the subsequent fall, at least from the 2nd to the 4th day, increases.

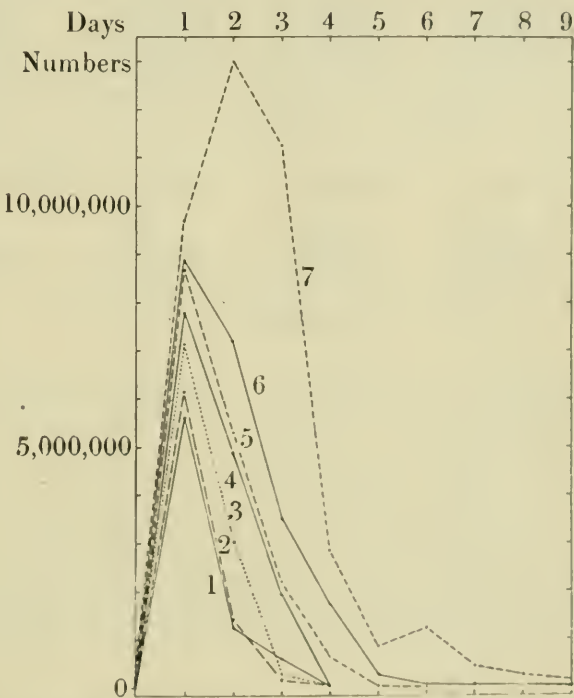


Chart 23. Showing the rate of multiplication in meat extract to which different quantities of glucose have been added. Tube 6 contains 0.1 % glucose; tube 5, 0.5 %; tube 4, 1 %; tube 3, 2.5 %; tube 2, 5 %; tube 1, 10 %; tube 7 contains no glucose and acts as a control.

Series IV was a repetition of part of Series II in order to ascertain the effect of small daily additions of food material, or glucose, or both, to cultures containing glucose.

Four tubes each containing meat extract 1.0 c.c., N/10 soda 0.08 c.c., distilled water 2.92 c.c. and glucose (5 per cent. in distilled water) 1.0 c.c.

were prepared, sterilised by boiling, inoculated with an emulsion of *S. aureus* in distilled water, and incubated at 33° C.

To tube I were added daily two drops of concentrated meat extract (100 c.c. meat extract evaporated to 10 c.c. and 10 c.c. of distilled water), to tube II two drops of concentrated meat extract with glucose (100 c.c. meat extract evaporated to 10 c.c. and 10 c.c. of 25 per cent. glucose in distilled water), and to tube III one drop of 5 per cent. glucose in distilled water. To tube IV which acted as a control no additions were made.

No. at beginning Tube of experiment		22 hours	48 hours	76 hours	4 days	5 days	
I	2024	5,392,000	2,176,000	2,688,000	2,120,000	576,000	
II	—	4,896,000	2,152,000	2,563,000	968,000	188,000	
III	—	6,608,000	2,036,000	616,000	412,000	224,000	
IV	—	5,824,000	2,640,000	2,024,000	1,048,000	2,184,000	
		6 days	7 days	8 days	9 days	10 days	11 days
I	356,000	160,000	244,000	232,000	784,000	2,784,000	
II	144,000	64,000	84,000	1,188,000	2,816,000	4,312,000	
III	640,000	648,000	450,000	176,000	—	224	
IV	568,000	428,000	200,000	28,000	6,500	432	
		12 days	13 days	14 days	15 days	17 days	19 days
I	3,792,000	5,952,000	4,504,000	4,744,000	952,000	228,000	
II	3,688,000	3,824,000	3,176,000	2,112,000	1,176,000	756,000	
III	154	27	17	8	10	41	
IV	87	9	4	6	30	32	
		21 days	23 days	26 days	28 days	31 days	35 days
I	1,752	223	161	154	13	77	
II	576,000	940,000	788,000	612,000	185,000	68,000	
III	2	18	29	7	30	6	
IV	100	103	233	176	248	152	
		39 days	41 days	44 days	48 days	52 days	
I	0	0	0	0	0		
II	103,000	29,500	0	0	0		
III	12	0	0	0	0		
IV	148	319	275	98	110		

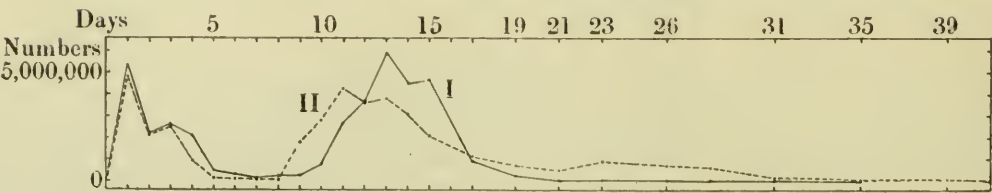


Chart 24. Showing the rate of multiplication in tubes of meat extract to which 1 % glucose was added, and to which daily additions of meat extract (I) and meat extract and glucose (II) were made.

In tube I, with daily additions of meat extract, after the primary rise the numbers fell to a moderately low level and then rose again to nearly the same level as in the primary rise. Subsequently a rapid fall to a low level



occurred and the culture was dead by the 39th day. In tube II with daily additions of meat extract and glucose the course of events was similar, but the fall after the secondary rise was slower, and the culture was dead on the 44th day. In tube III with daily additions of glucose the secondary rise was very slight, but very small numbers remained alive till the 39th day. In tube IV to which no addition was made there was a slow fall after the primary rise, and small oscillations subsequently. The culture was still alive on the 52nd day.

Series I, cultures 1, 2, 3, 4, Series II, culture 3, Series IV, culture 4, were similar in composition and Series III, culture 4, contained the same ingredients in the same proportions. The cultures in Series I, II and III behaved in the same manner, the organisms being dead in about seven to ten days, but in the cultures of Series IV the organisms after reaching a very low level on the 17th day, multiplied to a small extent and were still alive on the 52nd day. This perhaps indicates that if some of the organisms survive the critical period life may be prolonged for a very considerable time. The experiments described in Series II, culture 5, and in Series IV, culture 1, were very similar and indicate that in spite of the presence of glucose the daily addition of small quantities of meat extract prolongs the life of the culture; similarly Series II, culture 6, and Series IV, culture 2, show that in spite of the presence of glucose the daily addition of small quantities of meat extract and glucose prolongs the life of the culture. Series II, culture 7, and Series IV, culture 3, show that the daily addition of small quantities of glucose does not result in the rapid death of the culture.

SECTION XVII. *The effects of adding living organisms of the same species to growing cultures.*

Several series of experiments, of which four are quoted, were carried out in order to ascertain the effects of adding at different times varying numbers of *S. aureus* to cultures already growing in meat extract media. As it is necessary in such experiments to estimate the numbers at frequent intervals a low concentration of meat extract was employed so as to avoid very prolonged observations, and the errors liable to be introduced in counting large numbers. The strain of *S. aureus* employed had been isolated recently from pus.

*Exp. 1.* In the first experiment five tubes, each containing meat extract 0.5 c.c., N/10 soda 0.04 c.c. and distilled water 4.46 c.c., were employed. All the tubes received at the same time a primary inoculation of a drop of an emulsion of the coccus in distilled water, and all except tube A, which was used as a control, received at some time a second inoculation of a drop of a freshly prepared emulsion of the same coccus. The tubes were kept in an incubator at 37° C., but were taken out for a short time on each occasion on which subcultures were made from them.

Tube	Count before incubation	Cocci added		Cocci added			Cocci added
		1.75 hours	3.5 hours	6.25 hours	6.5 hours	8 hours	8.5 hours
A	2216	—	—	863,000	—	2,092,000	—
B	2552	1592	—	786,000	—	2,300,000	—
C	2592	—	1465	818,000	—	2,276,000	—
D	2320	—	—	836,000	2554	2,695,000	—
E	3004	—	—	848,000	—	2,384,000	1394
Mean	—	—	—	830,000	—	2,349,000	—
	15.75 hours	17 hours	20 hours	22 hours	26 hours	44 hours	68 hours
A	7,840,000	6,608,000	5,784,000	5,672,000	5,344,000	488,000	220,000
B	7,304,000	6,224,000	5,816,000	5,698,000	5,624,000	447,000	211,000
C	7,976,000	7,120,000	6,040,000	6,024,000	5,480,000	556,000	208,000
D	7,176,000	7,192,000	5,564,000	5,660,000	5,280,000	592,000	242,000
E	7,656,000	6,872,000	6,232,000	5,696,000	5,296,000	536,000	187,500
Mean	7,590,000	6,803,000	5,887,000	5,750,000	5,405,000	524,000	213,000

It will be seen that in this experiment the addition of small numbers of cocci at different times between 1.75 and 8.5 hours after the beginning of incubation exerted no influence which could be determined by the methods employed.

The slow rate of fall in numbers between the 15th and 26th hours was probably due to cooling of the tubes owing to frequent removals from the incubator in order to prepare dilutions for subcultures.

Exp. 2. In this series six tubes were used containing a medium of the same composition as in Exp. 1. In order to maintain an approximately equal temperature during the whole period the tubes were kept throughout in a water-bath at 37° C.

Tube	Count before incubation	Cocci added		Cocci added		2.5 hours
		0.5 hour	0.5 hour	1.5 hours	1.5 hours	
A	2896	2752	—	4112	—	9,984
B	2672	2768	3712	7824	—	12,960
C	2656	2920	—	4008	2624	10,944
D	2808	2776	—	4248	—	10,744
E	2720	2712	—	4002	—	11,080
F	2744	2800	—	4320	—	9,854
Mean	—	—	—	—	—	10,927
	Cocci added	Cocci added		Cocci added		Cocci added
	2.5 hours	5 hours	5 hours	6.5 hours	7.75 hours	7.75 hours
A	—	2,680,000	—	1,160,000	2,912,000	—
B	—	292,000	—	1,216,000	3,016,000	—
C	—	320,000	—	1,104,000	2,856,000	—
D	1808	292,000	—	1,228,000	2,720,000	—
E	—	342,000	1502	1,368,000	3,188,000	—
F	—	246,000	—	1,196,000	2,776,000	1672
Mean	—	293,000	—	1,212,000	2,911,000	—
	10 hours	12 hours	14 hours	16 hours	17.5 hours	
A	4,904,000	5,508,000	6,298,000	6,144,000	5,808,000	
B	4,960,000	5,932,000	6,040,000	6,008,000	5,208,000	
C	4,600,000	6,060,000	6,650,000	5,948,000	5,768,000	
D	5,186,000	6,443,000	7,050,000	6,816,000	6,634,000	
E	4,736,000	6,280,000	6,699,000	6,600,000	6,146,000	
F	4,104,000	5,982,000	6,891,000	6,080,000	5,820,000	
Mean	4,738,000	6,034,000	6,604,000	6,266,000	5,596,000	



In this experiment also the addition of small numbers of cocci seemed to have very little influence on the numbers present at various times, except in the early stages of tube *B* before rapid multiplication had begun.

*Exp. 3.* In this experiment four tubes were used, each containing meat extract 0.25 c.c., *N*/10 soda 0.02 c.c. and distilled water 4.73 c.c., and the tubes were kept throughout in the water-bath at 37° C. Half an hour after the commencement of incubation a small number of cocci were added to tube *B*, and a large number to tube *D*. A small number was added to tube *C* after 4.5 hours.

Tube	Count before incubation	0.5 hour	Cocci added				
			0.5 hour	2.5 hours	3 hours	3.5 hours	
<i>A</i>	905	897	—	1,600	2,676	4,024	
<i>B</i>	923	1047	2,369	3,828	4,328	4,996	
<i>D</i>	922	943	19,089	21,440	23,968	37,312	
<i>C</i>	794	805	—	1,152	2,028	2,336	

	4 hours	4.5 hours	Cocci added			
			4.5 hours	5.5 hours	6.5 hours	7.75 hours
<i>A</i>	5,984	20,000	—	63,000	114,500	428,000
<i>B</i>	7,768	16,600	—	53,500	112,000	336,000
<i>D</i>	50,944	201,500	—	600,000	980,000	1,972,000
<i>C</i>	3,640	10,800	2784	38,000	82,000	284,000

	8.75 hours	9 hours	10.25 hours	11.25 hours	12.25 hours	28 hours
<i>A</i>	606,000	1,128,000	2,128,000	2,696,000	3,264,000	1,716,000
<i>B</i>	594,000	828,000	1,544,000	2,748,000	3,124,000	1,708,000
<i>D</i>	2,424,000	2,572,000	3,216,000	3,772,000	3,064,000	1,564,000
<i>C</i>	548,000	717,000	1,488,000	2,680,000	3,316,000	1,604,000

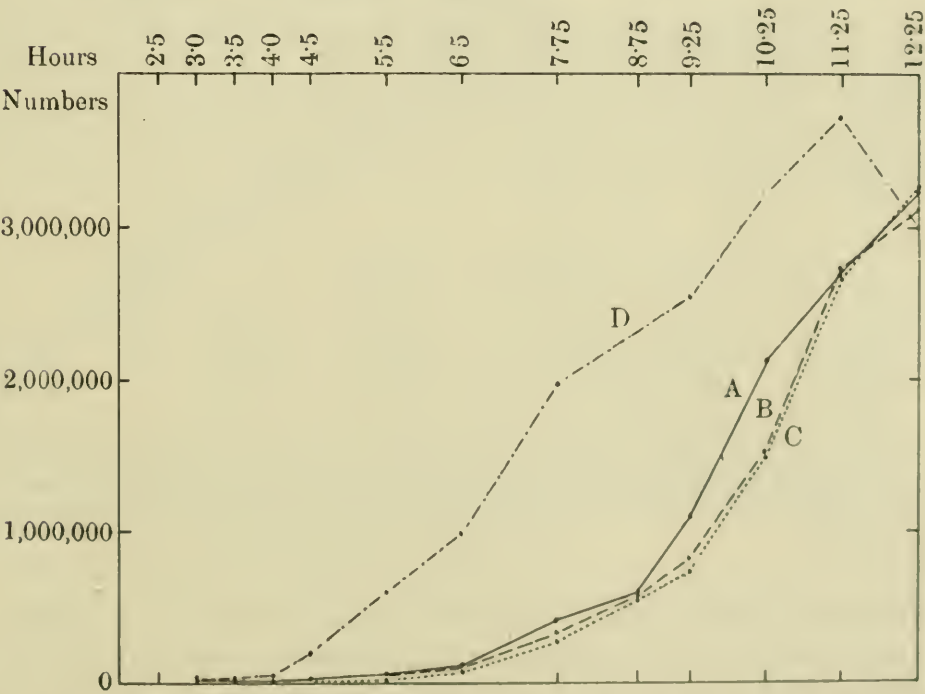


Chart 25. Showing the results of adding small numbers of cocci to growing cultures in tube *B* after 0.5 hour and to tube *C* after 4.5 hours, and larger numbers to tube *D* after 0.5 hour's incubation at 37° C., as compared with the control tube *A*.

It will be seen that in tube *D*, to which a large number of cocci were added within the lag period, the increase in numbers was most rapid. Little difference in the rate of growth in the other three tubes, the control *A*, and the tubes *B* and *C*, to which small numbers were added, could be detected. The experiment seems to indicate that while the addition of small numbers has an inappreciable influence on the numbers present at any period of growth, the addition of a large number, at any rate within the lag period, exerts a considerable influence.

*Exp. 4.* In this experiment three tubes, containing a medium of the same composition as in *Exp. 3*, were employed, and a large number of cocci added to tube *B* 0.75 hour, and to tube *C* 4 hours after the commencement of incubation.

Tube	Count before incubation	0.75 hour	Cocci added 0.75 hour	3 hours	4 hours	Cocci added 4 hours
A	1504	1372	—	2,592	18,400	—
B	1568	1696	27,872	30,656	307,200	—
C	1512	1536	—	2,632	20,200	9212
	5 hours	6 hours	8 hours	9 hours	10 hours	11 hours
A	35,200	138,000	604,000	1,320,000	3,992,000	4,868,000
B	1,008,000	2,076,000	3,992,000	4,928,000	5,380,000	5,496,000
C	45,600	164,800	1,348,000	3,528,000	4,800,000	5,252,000
	12 hours	13 hours	14 hours	27 hours	52 hours	
A	5,212,000	5,386,000	5,396,000	928,000	432,000	
B	5,600,000	5,624,000	5,424,000	1,008,000	536,000	
C	5,480,000	5,420,000	5,312,000	882,000	480,000	

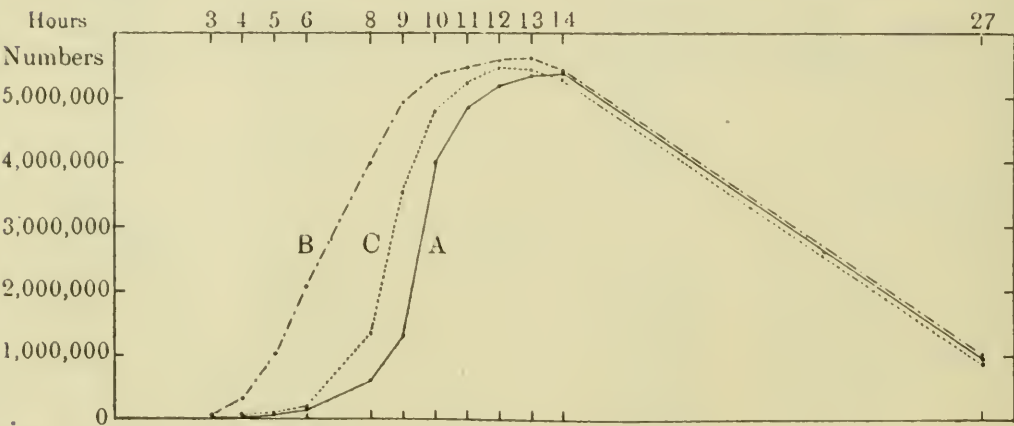


Chart 26. Showing the results of adding large numbers of cocci to growing cultures in tube *B* after 0.75 hour's and in tube *C* after 4 hours' incubation at 37° C., as compared with the control tube *A*.

It will be seen that in both *B* and *C* the numbers rose more rapidly than in the control tube *A*, showing that the addition of large numbers, at least within the first few hours of incubation, accelerates the rise in numbers.



SECTION XVIII. *The distribution of S. aureus in meat extract cultures.*

The experiments described in this section were undertaken in order to ascertain roughly the distribution of *S. aureus* in meat extract cultures after various periods of incubation at 37° C. Large tubes containing meat extract 2 c.c., *N*/10 soda 0.16 c.c. and distilled water 7.84 c.c. were employed. The strain of *S. aureus* used had been isolated freshly from pus.

(A) In one series the cultures were shaken once daily, as in most of the experiments described in this paper. Two samples were taken daily with a small pipette, containing 0.01 c.c. up to a diamond mark, the first from the upper part of the culture with as little disturbance as possible, and the second after thorough shaking. After dilution subcultures were made in the usual manner.

	Immediately after inoculation	44 hours	47 hours	70 hours	94 hours	118 hours
Before shaking	616	8,112,000	10,216,000	9,408,000	8,016,000	5,744,000
After shaking	—	9,600,000	12,416,000	10,816,000	9,504,000	7,424,000
		142 hours	166 hours	190 hours	214 hours	
		2,576,000	856,000	322,000	170,000	
		3,112,000	976,000	405,000	215,000	

It will be seen that 24 hours after each shaking about 15 per cent. of the living organisms had fallen towards the bottom of the tube, the majority of them probably forming the deposit.

(B) In another series of experiments the cultures were left undisturbed in the incubator until the time of examination.

Six tubes inoculated at the same time and incubated at 37° C. were employed. Samples from the upper part of the first culture tube before and after shaking were examined after 18 hours' incubation, from the second, third, fourth, fifth and sixth tubes after 2, 3, 4, 5 and 6 days' incubation respectively.

	Tube I		Tube II	Tube III	Tube IV	Tube V	Tube VI
	After inocula- tion	18 hours	48 hours	72 hours	96 hours	120 hours	144 hours
Before shaking	584	7,680,000	8,156,000	7,792,000	7,208,000	2,416,000	1,680,000
After shaking	—	8,880,000	10,736,000	10,976,000	8,992,000	3,132,000	2,080,000

It will be seen that after 48 hours' incubation about 25 per cent. of the living organisms had fallen to the bottom, but that subsequently the proportion at the bottom remained about the same.

(C) Effects of centrifugalisation. Cultures consisting of meat extract 1 c.c., *N*/10 soda 0.08 c.c. and water 3.92 c.c. were prepared in centrifugal tubes. After 22 hours' incubation at 37° C. a sample was taken from the upper part of a culture, the tube centrifugalised for one hour, and another sample taken from the top. The first sample showed 7,904,000 colonies, and the second 125,000 colonies, indicating that 98.5 per cent. of the organisms occurring

near the surface had been driven down. The upper 3 c.c. of the fluid, which were quite clear, were pipetted off, and again incubated for 25 hours. Subcultures from the top before shaking showed 6,848,000 colonies, and after shaking 8,176,000 colonies. The cocci remaining in the upper layers therefore multiplied in the same manner as cocci inoculated into fresh medium, and about 16 per cent. settled to the bottom.

Another culture incubated for 48 hours was centrifugalised for 3 hours. Before centrifugalisation subcultures from the top showed 11,232,000 colonies, and after, when the fluid was clear, 2344 colonies, or a reduction of 99.98 per cent. The upper 3 c.c. were pipetted off and incubated for 24 hours at 37° C. Before shaking the top showed 706,000 colonies, and after shaking 768,000 colonies.

#### SECTION XIX. *Meat extract and pancreas extract compared as media.*

In order to ascertain whether the mode of preparation of the extract influences the rate of growth of the organisms six equal portions from one heart were weighed out, ground up with sterile sand and triturated with 2.5 c.c. of distilled water to each gramme of meat. Six portions of bullock's pancreas were prepared in the same way. The heart preparations were labelled *H* 1, 2, 3, 4, 5, 6 and the pancreas preparations *P* 1, 2, 3, 4, 5, 6, and were treated in the following manner.

- H* 1 and *P* 1. Boiled immediately after preparation for 10 minutes, boiled next day, filtered through filter paper and again boiled.
- H* 2 and *P* 2. Autoclaved immediately after preparation for 20 minutes, filtered next day, and again autoclaved.
- H* 3 and *P* 3. 2 per cent. chloroform added and incubated for 24 hours at 37° C., boiled and filtered and again boiled.
- H* 4 and *P* 4. 2 per cent. chloroform added and incubated for 24 hours at 37° C., autoclaved and filtered and again autoclaved.
- H* 5 and *P* 5. Incubated for 24 hours at 37° C. without chloroform, boiled and filtered and again boiled.
- H* 6 and *P* 6. Incubated for 24 hours at 37° C. without chloroform, autoclaved and filtered and again autoclaved.

In the preparations labelled *H* 5, 6 and *P* 5, 6 putrefactive organisms had grown, and before sterilisation the preparations were turbid and foul smelling.

From each extract two tubes were prepared, in one the extract was diluted with distilled water (*e.g.* *H* 1) and in the other neutralised to neutral red with *N*/10 soda and diluted (*e.g.* *H* 1 A). The composition of these tubes is given in the following table. It will be noticed that the pancreas extract when incubated for 24 hours at 37° C., whether with or without chloroform, requires a relatively large quantity of soda to neutralise it.

Two such sets of media were prepared; one was inoculated in the usual manner with *S. aureus* and the other with *B. coli*.



	Meat extract	N/10 soda	Distilled water		Pancreas extract	N/10 soda	Distilled water
<i>H</i> 1	1.0 c.c.	0	1.0	<i>P</i> 1	1.0 c.c.	0	1.0
1 A	1.0	0.05	0.95	1 A	1.0	0.05	0.95
<i>H</i> 2	1.0	0	1.0	<i>P</i> 2	1.0	0	1.0
2 A	1.0	0.05	0.95	2 A	1.0	0.05	0.95
<i>H</i> 3	1.0	0	1.0	<i>P</i> 3	1.0	0	1.0
3 A	1.0	0.075	0.925	3 A	1.0	0.25	0.75
<i>H</i> 4	1.0	0	1.0	<i>P</i> 4	1.0	0	1.0
4 A	1.0	0.1	0.9	4 A	1.0	0.375	0.625
<i>H</i> 5	1.0	0	1.0	<i>P</i> 5	1.0	0	1.0
5 A	1.0	0.1	0.9	5 A	1.0	0.225	0.775
<i>H</i> 6	1.0	0	1.0	<i>P</i> 6	1.0	0	1.0
6 A	1.0	0.125	0.875	6 A	1.0	0.35	0.65

Except in the modified form quoted later (p. 195), these experiments have not been repeated and therefore too much stress cannot be laid on them, but attention may be called to the following points.

*Meat extract.* There is no appreciable difference between the results obtained with fresh meat extract sterilised by boiling (*H* 1, 1 A) and by autoclaving (*H* 2, 2 A). The curve is higher and more prolonged with meat extract incubated with chloroform and sterilised by boiling (*H* 3, 3 A), the unneutralised sample (*H* 3) showing a curve like that produced when small quantities of N/10 hydrochloric acid have been added to fresh meat extract. In the samples of meat extract incubated with chloroform, and sterilised by autoclaving (*H* 4, 4 A) the neutralised specimen (*H* 4 A) produces a curve similar to *H* 3 A, but in the unneutralised specimen (*H* 4) the curve though prolonged is relatively low. In the samples incubated without chloroform and sterilised by boiling (*H* 5, 5 A) the curves resemble those produced with fresh extract, but the maximum growth in the whole series was obtained with similar samples sterilised by autoclaving (*H* 6, 6 A). In the two samples incubated without chloroform growth of putrefactive organisms had occurred.

*Pancreas extract.* Growth was considerably greater in the sample of fresh pancreas extract sterilised by autoclaving (*P* 2, 2 A) than in the sample sterilised by boiling (*P* 1, 1 A). By far the greatest multiplication took place in the unneutralised specimen of extract incubated with chloroform and sterilised by boiling (*P* 3), but in the neutralised specimen of the same sample it was not so great. In the samples incubated with chloroform and sterilised by autoclaving (*P* 4, 4 A) the results most closely resemble those obtained with fresh autoclaved samples (*P* 2, 2 A). It is of interest to note that multiplication was very slight during the first day in the neutralised specimen (*P* 4 A). The least growth in this series was obtained with samples incubated without chloroform (*P* 5, 5 A, 6, 6 A).

It is evident that under all the conditions of these experiments, except those in which putrefactive organisms had grown (*H* 5, 6, *P* 5, 6), pancreas extract is a much better medium for the growth of *Staphylococci* than meat

Cultures inoculated with <i>Staphylococcus</i> .										Fluid in tube, c.c.	
	20 hours	2 days	3 days	4 days	5 days	6 days	7 days	8 days	9 days	10 days	37 days
<i>H</i> 1	7,488,000	12,336,000	9,856,000	3,968,000	948,000	644,000	628,000	1,032,000	1,508,000	1,736,000	1,660,000
1 A	9,792,000	14,848,000	10,528,000	2,032,000	972,000	868,000	1,064,000	1,192,000	896,000	720,000	1,236,000
2	8,424,000	12,944,000	9,760,000	3,600,000	3,284,000	2,392,000	936,000	1,144,000	971,000	1,576,000	1,184,000
2 A	10,752,000	13,472,000	9,264,000	8,304,000	2,432,000	—	512,000	536,000	596,000	1,168,000	3,376,000
3	8,296,000	8,976,000	8,432,000	11,696,000	15,984,000	12,992,000	9,288,000	4,336,000	3,984,000	4,240,000	4,585,000
3 A	10,192,000	12,112,000	14,032,000	14,832,000	13,232,000	4,968,000	2,624,000	2,624,000	2,063,000	1,856,000	4,328,000
4	7,976,000	5,816,000	5,936,000	6,704,000	6,816,000	6,584,000	5,404,000	5,824,000	3,776,000	3,152,000	5,936,000
4 A	7,992,000	13,280,000	12,512,000	12,896,000	12,368,000	5,264,000	1,916,000	3,288,000	4,208,000	3,584,000	3,152,000
5	8,264,000	12,656,000	10,592,000	5,504,000	3,288,000	2,128,000	2,372,000	2,704,000	2,496,000	2,208,000	2,448,000
5 A	9,752,000	18,224,000	12,144,000	5,968,000	3,496,000	2,708,000	2,096,000	1,904,000	1,632,000	1,424,000	2,472,000
6	7,216,000	20,624,000	20,144,000	21,056,000	16,848,000	8,176,000	4,784,000	3,816,000	2,762,000	2,608,000	7,456,000
6 A	8,856,000	19,136,000	19,392,000	9,312,000	6,112,000	3,120,000	1,616,000	1,856,000	1,611,000	1,712,000	7,505,000
<i>P</i> 1	15,600,000	18,720,000	18,048,000	16,496,000	12,640,000	7,184,000	4,944,000	3,000,000	1,600,000	—	3,312,000
1 A	14,892,000	16,096,000	15,660,000	17,776,000	14,144,000	9,120,000	6,944,000	5,184,000	2,704,000	—	3,216,000
2	17,024,000	24,656,000	23,712,000	23,684,000	14,136,000	10,304,000	7,048,000	11,760,000	11,536,000	—	5,982,000
2 A	20,608,000	26,920,000	30,624,000	26,244,000	14,720,000	6,200,000	3,056,000	6,048,000	5,120,000	—	—
3	40,416,000	51,984,000	39,520,000	22,288,000	13,872,000	11,648,000	7,856,000	13,856,000	10,800,000	—	8,912,000
3 A	31,888,000	29,168,000	21,750,000	12,800,000	7,456,000	6,048,000	5,840,000	5,440,000	9,044,000	—	6,656,000
4	13,888,000	18,112,000	26,782,000	22,384,000	11,248,000	6,560,000	4,304,000	5,920,000	2,304,000	—	2,400,000
4 A	194,000*	17,114,000	32,096,000	30,832,000	11,968,000	9,696,000	5,248,000	8,992,000	7,584,000	—	5,904,000
5	5,472,000	11,024,000	10,544,000	9,856,000	8,144,000	7,520,000	4,592,000	6,704,000	5,536,000	—	2,080,000
5 A	8,512,000	8,272,000	8,388,000	9,216,000	8,000,000	7,440,000	5,616,000	4,944,000	5,088,000	—	4,176,000
6	2,304,000	5,088,000	8,480,000	10,752,000	10,816,000	10,752,000	5,080,000	2,848,000	5,792,000	—	2,688,000
6 A	2,780,000*	6,880,000	6,752,000	7,408,000	7,840,000	4,800,000	5,328,000	3,632,000	4,288,000	—	3,488,000

\* Colonies very small after 24 hours' growth.



extract. The rate of growth was greatest in unneutralised pancreas extract incubated with chloroform and sterilised by boiling (*P* 3). On the other hand, an inhibition of multiplication for the first 24 hours took place in the neutralised specimen incubated with chloroform and sterilised by autoclaving (*P* 4 A). Further investigations on this subject are being carried on.

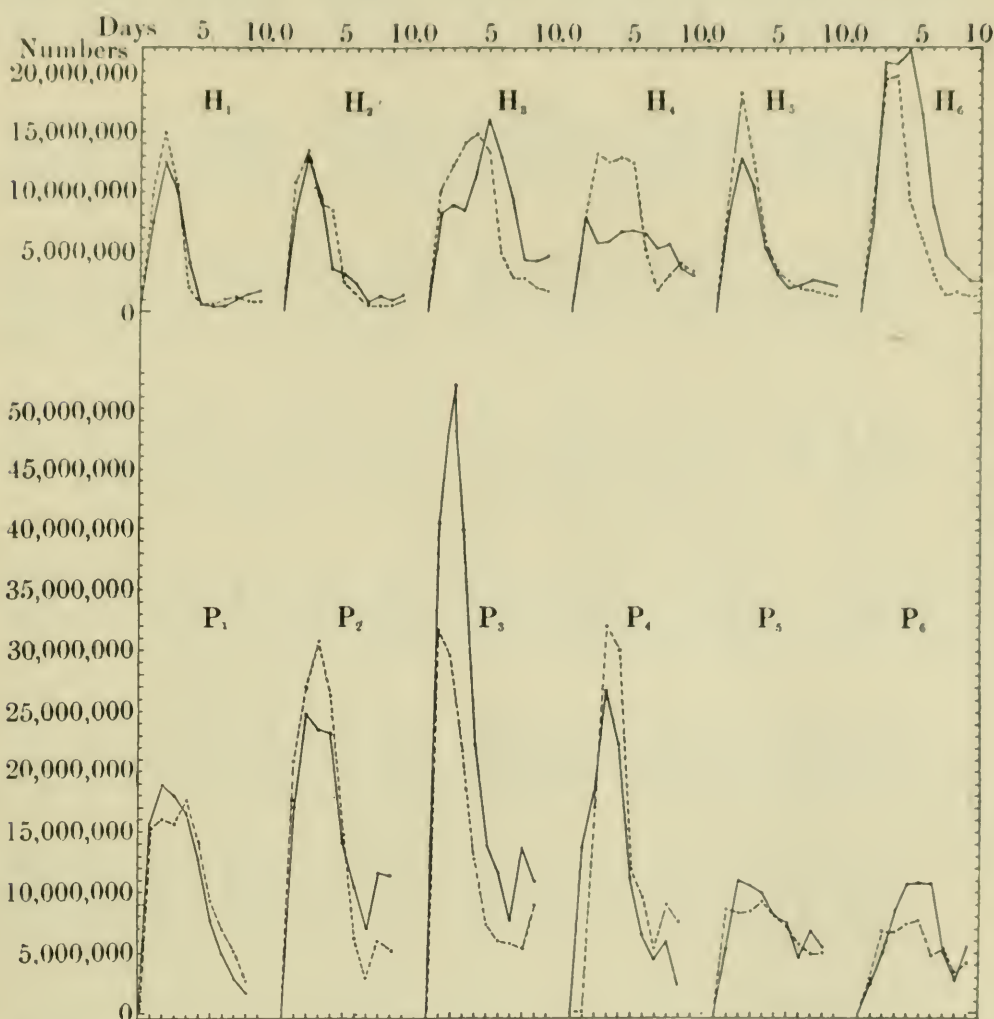


Chart 27. Showing the rate of multiplication of *S. aureus* in samples of meat extract (*H*) and pancreas extract (*P*) prepared in different ways. The continuous line indicates unneutralised and the broken line neutralised samples.

In spite of great reduction in the quantity of the fluid in each tube by the removal of samples and evaporation, in all cases the organisms were alive on the 37th day.

In many features the results with *B. coli* closely resemble those with *Staphylococci*.

*Meat extract.* With fresh meat extract the curves are similar whether sterilisation was by boiling (*H* 1, 1 A) or by autoclaving (*H* 2, 2 A). Meat extract incubated with chloroform and boiled (*H* 3) gives a moderately high figure with less of the delay noticed in the cultures sown with *Staphylococci*. Extract incubated with chloroform and autoclaved (*H* 4, 4 A) gives

Cultures inoculated with *B. coli*.

	26 hours	2 days	3 days	4 days	5 days	6 days	7 days	9 days	11 days	15 days	Fluid in tube, c.c.
<i>H</i> 1	5,904,000	10,592,000	10,176,000	8,112,000	4,640,000	3,136,000	2,176,000	3,104,000	2,896,000	1,088,000	.47
1 A	7,088,000	12,128,000	9,248,000	5,072,000	3,936,000	3,238,000	2,096,000	2,032,000	2,016,000	800,000	.73
2	5,440,000	11,472,000	6,528,000	1,904,000	1,488,000	—	992,000	768,000	760,000	288,000	.6
2 A	8,176,000	12,080,000	6,592,000	3,632,000	2,672,000	2,720,000	1,856,000	2,566,000	1,536,000	992,000	.66
3	5,056,000	12,760,000	9,248,000	8,896,000	3,952,000	2,960,000	2,784,000	3,056,000	1,968,000	688,000	.65
3 A	6,288,000	6,904,000	10,176,000	3,872,000	2,976,000	1,888,000	2,080,000	1,904,000	1,264,000	144,000	.75
4	2,400,000	4,912,000	7,008,000	9,200,000	6,512,000	4,368,000	3,872,000	5,200,000	6,560,000	10,096,000	.22
4 A	7,840,000	7,084,000	3,856,000	2,512,000	808,000	2,720,000	2,544,000	2,192,000	2,096,000	600,000	.62
5	4,656,000	12,624,000	10,352,000	4,704,000	3,424,000	4,000,000	2,048,000	2,096,000	1,280,000	1,507,000	.43
5 A	4,960,000	11,360,000	7,152,000	6,352,000	4,432,000	2,288,000	2,144,000	3,360,000	1,120,000	504,000	.43
6	4,016,000	9,648,000	11,296,000	8,640,000	6,576,000	5,536,000	4,800,000	4,240,000	3,968,000	5,152,000	.31
6 A	5,568,000	10,514,000	7,936,000	4,928,000	4,080,000	4,014,000	4,400,000	4,848,000	2,912,000	4,112,000	.54
<i>P</i> 1	5,088,000	14,064,000	17,024,000	15,776,000	10,544,000	6,834,000	8,128,000	7,904,000	4,576,000	4,240,000	.73
1 A	6,624,000	15,264,000	15,696,000	12,120,000	11,312,000	8,960,000	9,344,000	5,238,000	4,416,000	2,720,000	.7
2	7,248,000	17,808,000	17,888,000	16,912,000	12,768,000	8,594,000	7,936,000	9,360,000	9,600,000	8,805,000	.15
2 A	7,390,000	17,440,000	18,736,000	19,420,000	12,912,000	10,720,000	7,840,000	11,792,000	—	—	—
3	19,820,000	7,258,000	3,904,000	3,400,000	5,248,000	4,096,000	3,456,000	9,792,000	7,600,000	3,488,000	.1
3 A	16,848,000	7,280,000	2,064,000	1,520,000	2,048,000	2,720,000	1,520,000	8,688,000	6,304,000	7,808,000	.62
4	12,688,000	15,168,000	9,552,000	4,416,000	3,200,000	1,904,000	2,096,000	8,400,000	4,800,000	6,176,000	.58
4 A	16,048,000	8,544,000	4,016,000	2,288,000	2,240,000	2,016,000	1,968,000	6,237,000	4,512,000	4,896,000	.68
5	5,008,000	4,288,000	3,056,000	1,232,000	976,000	928,000	1,344,000	1,952,000	5,132,000	2,576,000	.73
5 A	4,032,000	3,000,000	2,304,000	1,152,000	1,088,000	1,306,000	1,552,000	2,544,000	4,896,000	3,618,000	.53
6	5,488,000	640,000	1,536,000	1,360,000	1,072,000	1,072,000	736,000	1,680,000	1,216,000	704,000	.72
6 A	4,752,000	336,000	1,112,000	1,168,000	688,000	1,040,000	544,000	1,072,000	960,000	336,000	.48



ow figures with, in the case of the unneutralised sample (*H* 4), considerable delay in reaching the maximum. Good growths were obtained in the samples (*H* 5, 6) in which putrefactive organisms had grown. *Pancreas extract*. *B. coli* grows better in fresh pancreas extract than in meat extract, and better in the autoclaved (*P* 2) than in the boiled sample (*P* 1). Pancreas extract incubated with chloroform and boiled (*P* 3) gives the most rapid growth of the whole series, but growth in the autoclaved sample (*P* 4) is not better

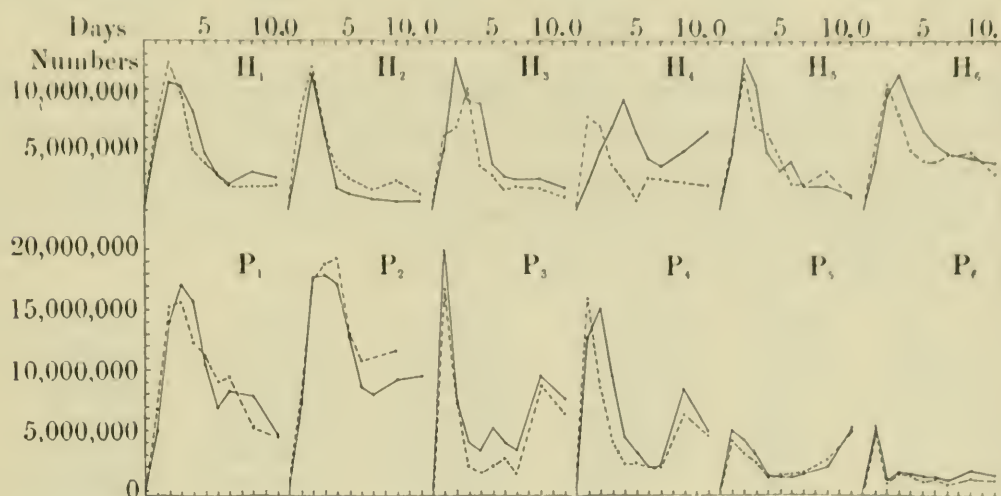


Chart 28. Showing the rate of multiplication of *B. coli* in samples of meat extract (*H*) and pancreas extract (*P*) prepared in different ways. The continuous line indicates unneutralised and the broken line neutralised samples.

than in the fresh extract. In the neutralised specimen (*P* 4 A) no early inhibition, such as occurred with *Staphylococci*, took place; in fact very rapid early growth occurred. Multiplication was comparatively very small in the samples (*P* 5, 6) in which putrefactive organisms had grown.

As in the case of *S. aureus* unneutralised pancreas extract incubated with chloroform and boiled (*P* 3) is the medium which gives the most rapid growth, but in the case of *B. coli* its superiority over pancreas extracts treated in other ways is not so marked.

## SECTION XX. *Further experiments with media made from incubated ox pancreas.*

To 100 grms. of fresh, finely minced ox pancreas 250 c.c. of distilled water and 5 c.c. of chloroform were added, and the mixture incubated for 24 hours at 37° C. After thorough shaking the contents of the flask were divided into two portions. One portion, *P* 3, was steamed for 20 minutes, filtered, and the filtrate boiled. The other portion, *P* 4, was autoclaved, filtered and the filtrate again autoclaved. 10 c.c. of the former required 3.0 c.c. of *N*/10 soda, and 10 c.c. of the latter 3.2 c.c. *N*/10 soda to bring the reaction to the neutral point of neutral red.

From each medium a series of tubes containing different quantities of *N/10* soda and of distilled water were prepared.

	<i>P</i> 3	<i>N/10</i> soda	Distilled water		<i>P</i> 4	<i>N/10</i> soda	Distilled water
<i>A</i>	1	0	4	<i>A</i>	1	0	4
<i>B</i>	1	0.1	3.9	<i>B</i>	1	0.1	3.9
<i>C</i>	1	0.2	3.8	<i>C</i>	1	0.2	3.8
<i>D</i>	1	0.3	3.7	<i>D</i>	1	0.3	3.7
<i>E</i>	1	0.4	3.6	<i>E</i>	1	0.4	3.6
<i>F</i>	1	0.5	3.5	<i>F</i>	1	0.5	3.5

Each tube was inoculated with a drop of an emulsion in distilled water of *S. aureus* recently isolated from pus. The cultures were incubated at 37° C., plates prepared daily, and the colonies counted in the usual manner.

It will be noticed that the dilution of the pancreas extract is greater than in the experiments quoted in the last section.

	4 hours	24 hours	48 hours	3 days	4 days
<i>P</i> 3 <i>A</i>	39,712	29,644,000	43,328,000	42,048,000	31,498,000
<i>B</i>	16,032	26,624,000	42,528,000	41,334,000	35,904,000
<i>C</i>	4,592	25,376,000	40,320,000	39,712,000	33,472,000
<i>D</i>	2,112	22,880,000	41,608,000	41,728,000	37,120,000
<i>E</i>	1,968	5,136	19,328,000	35,904,000	38,464,000
<i>F</i>	1,024	3,696	317,000	12,576,000	15,056,000
	5 days	7 days	9 days	11 days	14 days
<i>P</i> 3 <i>A</i>	28,864,000	15,296,000	9,536,000	3,936,000	2,480,000
<i>B</i>	26,048,000	18,048,000	11,008,000	6,144,000	4,608,000
<i>C</i>	23,744,000	19,752,000	15,040,000	7,832,000	6,048,000
<i>D</i>	35,520,000	21,638,000	16,512,000	7,104,000	3,248,000
<i>E</i>	40,960,000	34,432,000	31,464,000	24,128,000	10,656,000
<i>F</i>	17,664,000	14,848,000	12,480,000	11,968,000	5,568,000
	4 hours	24 hours	48 hours	3 days	4 days
<i>P</i> 4 <i>A</i>	2296	10,544,000	20,800,000	23,936,000	26,816,000
<i>B</i>	1896	11,184,000	33,952,000	37,616,000	40,576,000
<i>C</i>	1480	134,000	27,392,000	32,256,000	37,192,000
<i>D</i>	1592	506	100,500	20,288,000	28,192,000
<i>E</i>	1432	10	1	0	0
<i>F</i>	1296	8	0	0	0
	5 days	7 days	9 days	11 days	14 days
<i>P</i> 4 <i>A</i>	30,784,000	21,642,000	16,512,000	13,696,000	6,752,000
<i>B</i>	43,072,000	32,832,000	25,728,000	19,072,000	12,715,000
<i>C</i>	40,064,000	34,600,000	25,088,000	16,960,000	9,472,000
<i>D</i>	34,652,000	32,320,000	24,384,000	19,264,000	8,672,000
<i>E</i>	0	0	0	0	0
<i>F</i>	0	0	0	0	0

Cultures were made from the tubes immediately after inoculation and the mean number of organisms found was 1520.

Though during the first few hours multiplication was greatest in the earlier specimens in the *P* 3 series after 24 hours' cultivation there was little difference between the results in *P* 3 *A*, *B* and *C*, and in Chart 29 the con-



tinuous line,  $x$ , represents the daily mean of these three cultures. The numbers in *P 3 D* fell more slowly during the earlier stages of the decline. In *P 3 E*, which was slightly alkaline, multiplication was slow during the first 24 hours, and the maximum was not attained until the fifth day. In *P 3 F* multiplication was very slow during the first 48 hours and a relatively low maximum was reached about the fifth day.

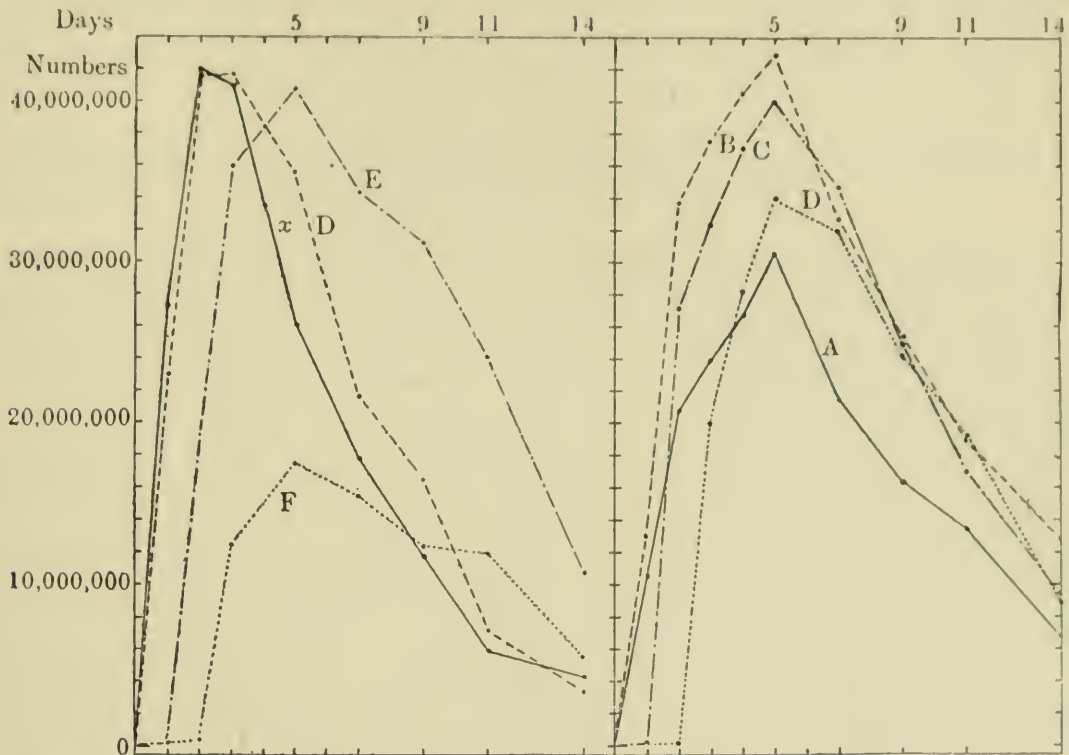


Chart 29. Showing on the left the results of growing cocci in *P 3* medium. The line  $x$  represents the mean of the three cultures *A*, *B*, *C* in which the reaction was acid. In *D* the reaction was neutral and in *E* and *F* slightly alkaline. On the right are shown the results of growing cocci in *P 4* medium. *A* was distinctly acid, *B* less acid and *C* still less acid. *D* was nearly neutral.

In the *P 4* series multiplication took place in the first four tubes, *A*, *B*, *C* and *D*, only, and all reached their maximum about the fifth day, the highest figures being attained in cultures *B* and *C*. In *P 4 C* multiplication was very slow during the first 24 hours, and in *P 4 D* during the first 48 hours. In the alkaline cultures *P 4 E* and *F* no multiplication occurred, and the organisms soon died.

In both these media *Staphylococci* multiply to an extraordinary extent provided that the quantity of soda added is not sufficient to bring the reaction beyond the neutral point of neutral red.

#### SECTION XXI. *The effects of accidental contamination.*

Though experiments in which accidental contamination occurred were soon abandoned some features worthy of record were observed. When cultures of *S. aureus* became contaminated with certain aerobic, spore-bearing bacilli

a sudden and unexpected decrease in the numbers of the cocci was noticed, even when the contaminating organisms were so few as to produce but one or two colonies on the agar subcultures. On the other hand on one occasion a great and unexpected increase in the numbers of coccus colonies was observed when the culture became contaminated with a small, diphtheroid bacillus. Instances have occurred in which the growth of a streptothrix or a mould did not appear to exert any influence on the growth of the cocci. It is evident, therefore, that the introduction into the medium of an organism of a different species may exert a great influence on the growth of *S. aureus*.

THE BEARING OF THESE EXPERIMENTS ON SOME OF THE PHENOMENA  
OBSERVED IN INFECTIVE DISEASES.

It has been suggested by Penfold (1914) that the "incubation period of infectious diseases may partly depend for its existence on bacterial lag." Some of the experiments described in this paper suggest the possibility that certain of the phenomena observed in connection with infective diseases depend, at least to some extent, on the available supply of food for the organisms.

It is evident that pathogenic bacteria living in the body cannot multiply without food, and that this food must in most cases be derived from the tissues. Though several factors doubtless operate in checking the multiplication of bacteria after they have gained entrance into the body their capacity to multiply must depend on their ability to procure food.

If the food is derived directly from healthy, living tissue, the supply is almost unlimited, but if it is a breakdown product then possibly the enzymes of the injured tissue as well as the enzymes of the bacteria play a part in its manufacture, and food in sufficient quantities to keep up a high rate of multiplication fails, unless the agents concerned in its production are acting efficiently. In a localised suppuration a variation in reaction or other result of local changes may produce conditions such as to inhibit partially the action of the enzymes.

Speculations such as these are suggested by certain resemblances between some of the phenomena observed in infective diseases and events in cultures.

(1) In disease an incubation period of longer or shorter duration invariably occurs. Its length probably depends on the virulence of the organisms and the size of the initial dose. In cultures the "lag" (or incubation period) can be abolished by using for inoculation rapidly multiplying organisms accustomed to the medium. In diseases associated with certain classes of organisms the incubation period may be greatly reduced experimentally by repeatedly passing the organism through the same species of animal. By this means the organism becomes accustomed to the medium (animal body) and its virulence, or capacity to grow in the tissues, is increased. In cultures, if the medium is not very suitable, many of the organisms die and a large inoculation is required to ensure growth. The form of the curve of growth depends to some extent on the numbers introduced (Section IV) and their source (Sections II, XIII).



Experimentally in animals a dose of a certain size is required, unless the organism is highly virulent, in order to secure the production of disease, and the course of the resulting disease depends to some extent on the size of the dose.

(2) In diphtheria the bacilli growing in the tonsillar crypts may be compared to organisms growing in test-tubes, and the numbers present at any time on the surface of the tonsils can be estimated roughly. Rapid multiplication of diphtheria bacilli occurs after the incubation period and for a longer or shorter time very great numbers are present. Subsequently the numbers decline until a low level has been reached. In carriers, who show few or no symptoms, the same phenomenon occurs, so that the process is independent of susceptibility or immunity to the toxins. The normal course of events in meat extract cultures is similar.

(3) The low level just mentioned often persists with slight variations for weeks or even months in diphtheria convalescents and carriers, and in the same manner small numbers of organisms remain alive in cultures and apparently exhibit slight oscillations in numbers. It often happens that one or more negative cultures from the tonsils are followed by positive cultures and this may happen on several occasions before the three consecutive negative cultures required for release are recorded.

(4) In typhoid, diphtheria and other carriers relapses occasionally occur, when the specific organisms again become numerous. These relapses are often preceded by slight deviations from normal health, in which catarrh is a prominent feature. These ailments which are often associated at the sites of infection with local changes, possibly resulting in an increase of material available as food to the specific organisms, appear to be due to intercurrent infections.

In cultures temporary multiplication may be induced by occasional small additions of food material.

(5) While some intercurrent infections appear to cause multiplication of the diphtheria bacilli in the tonsils of carriers, others, such as those associated with streptococci, may apparently cause their extermination. In cultures some accidental contaminations produce similar effects.

(6) Regular small additions of food material to cultures result in a high level being maintained or, if the additions are not sufficiently large, in the rate of decline being very slow. In some carriers large numbers of diphtheria bacilli persist for very long periods of time in the throat or nose, and perhaps in them there exist circumstances producing analogous results. Shearer (1917) showed that in nasal secretion "there is present some body, which greatly accelerates the growth of meningococcus on artificial culture medium," and Kligler (1919) investigated "saline washings of the nose of apparently healthy individuals." He states that "there were marked individual and specific differences. A given organism grew in the washings of one individual and not in those of another. Evidently the nasal secretions of some individuals contain substances, which actively stimulate growth."

(7) It is not uncommon to observe in both mild clinical cases and carriers a sudden fall from great numbers ending in the rapid disappearance of the diphtheria bacilli from the surface of the tonsils. In cultures, if the number of organisms inoculated is relatively large, the maximum is soon reached and the decline in numbers is very rapid owing to the speedy exhaustion of the food supply. The surprisingly rapid disappearance of the bacilli in the class of case mentioned may be due partly to the rapid exhaustion of the available food in the tonsillar crypts.

(8) The sequence of events in two individuals apparently infected from the same source may be very different. Whereas in one the symptoms may reach their full height in a few days, in the other the incubation period may be longer and the symptoms may not reach their height for several days. Presumably in the latter the early free multiplication of the organisms has been checked. Similar phenomena are noticed when equal doses of organisms are inoculated into neutral and slightly acid meat extract cultures.

(9) The acclimatisation experiments illustrate in cultures the phenomenon observed in streptococci of increase of virulence or capacity to grow in one species of animal (acid medium) simultaneously with loss of virulence for another species (alkaline medium).

Experiments on local immunity such as those carried out by Cobbett and Melsome (1896) on the ears of rabbits with *Streptococcus erysipelatus* might decide to what extent the exhaustion of food supply is a factor in conferring temporary local immunity. These workers showed that "an absolute local immunity had been conferred upon the parts directly affected by the first attack, unless the interval had been long enough to permit of the entire disappearance of all inflammatory thickening." On second inoculation they "could get no evidence of the invasion of these ears by streptococci." Transitory inflammation could, however, be produced by the inoculation of killed cocci or their poisonous products, showing that local resistance to the organisms is independent of local resistance to their toxins.

The speculations contained in this paragraph suggested themselves from time to time during the course of the work, but no attempt was made to prove them by animal experiments. Should they stimulate further research on the factors influencing the increase and decline in numbers of pathogenic bacteria in the tissues they will have fulfilled their purpose.

#### SUMMARY.

1. In dilute neutral meat extract cultures (without salt or peptone) inoculated with relatively small numbers of *S. aureus*, taken from agar cultures grown for 18 hours at 37° C. and incubated at 37° C., multiplication proceeds rapidly during the first day and more slowly on the second, when the maximum number, about 10,000,000 per standard loop (0.01 c.c.), is reached. Later the number of living organisms decreases at first rapidly, but later more slowly, until a low level is reached, which remains fairly constant or falls very slowly



for a long period. During the period of relative constancy small oscillations are observed. The curve produced on plotting out the daily counts may be regarded as a "standard."

2. The frequency with which the culture used for inoculation has been transplanted on agar slopes influences the growth on neutral meat extract. Several transplantations in rapid succession result in very rapid growth, a high maximum and a very rapid fall in the numbers. Less frequent transplantation over a long period seems to cause the maximum to be reached later than in the standard and the period of decline to be postponed.

3. In one series of experiments (Section III) the proportion of meat extract was varied in the different tubes employed. These experiments show that the greater the proportion of meat extract the greater is the multiplication, and the longer the period which elapses before the curve reaches its highest point, in fact the extent of multiplication appears to be closely related to the amount of meat extract present in the culture. The length of the period of rapid decline is also related to the amount of meat extract present.

4. The form of the curve of growth is influenced by the number of cocci inoculated. With a small inoculation into dilute neutral meat extract the maximum number of cocci present in the medium at any period does not usually exceed 10 to 12 millions per standard loop. If the initial dose greatly exceeds this figure multiplication proceeds relatively slowly for two days and subsequently there is a very rapid fall in the numbers. With an initial dose close to this figure a somewhat similar curve is produced, though the rate of fall is not so rapid. Much smaller doses produce "standard" types of curves.

5. Provided the numbers inoculated are small (50,000–50 per drop) the results after 24 hours' incubation in different experiments of the same kind are not materially affected.

6. If after the numbers have reached a low level small drops of concentrated meat extract, insufficient to cause appreciable dilution, are added to the culture further multiplication occurs, to some extent proportional to the amount of food material added. The fall in numbers, which follows the initial rise, is not due therefore to the accumulation of products, but seems to be caused mainly by the using up of food material.

7. By small regular additions of food material (concentrated meat extract) a definite concentration of *Staphylococci* can be maintained in a meat extract medium for a long period of time, and probably by suitable additions any desired concentration could be maintained. Accumulation of the products may gradually inhibit growth, but on this point the experiment gives little evidence.

8. Moderate dilution with distilled water at any stage of incubation has little effect. Events occur in the usual sequence, but the number of organisms in each standard drop is proportional to the dilution.

9. The incubation temperature has a great influence on the course of events in meat extract cultures of *S. aureus*. At 37° C. multiplication during the first 24 hours is very rapid, the maximum is attained on the second or third

day, and the numbers fall very rapidly. At 27° C. the maximum is attained on the fifth or sixth day, and is considerably greater than that attained at 37° C. The fall is rapid. At 17° C. multiplication is very slow during the first 48 hours, but is subsequently rapid, and the maximum, which is higher than that attained at 27° C., is reached on the eighth day. The decline in numbers is slow.

At 8 to 10° C. very slight multiplication, if any, occurs during the first 24 hours and subsequently the numbers steadily decline for at least 60 days.

At lower temperatures the numbers fall rapidly and the cultures die. At -1° C. the organisms were dead by the 19th day, at -6° C. by the 13th day, and at -10° C. by the 9th day.

10. If organisms such as *S. aureus*, *B. coli* or *B. pyocyaneus* are allowed to grow in meat extract medium at 37° C. until the numbers have reached a low level, and the tubes are then inoculated with the species originally present little or no multiplication takes place, but if one of the other organisms is inoculated multiplication of the added organisms occurs. If the cultures are sterilised by boiling before inoculation with fresh organisms the original strain or the others, when added, multiply. Boiling, therefore, appears to liberate some food for added organisms belonging to the strain which was originally present.

The growth of any of these organisms in the medium seems to remove most of the food for that species as well as a portion of the food substance used by other species, since in no case was the growth of the added species nearly so considerable in extent as in its primary cultures.

11. The effect of adding increasing quantities of *N/10* hydrochloric acid up to 0.3 c.c. to each 5 c.c. of the medium is to retard the growth of the cocci during the earlier stages of incubation, though subsequently rapid growth takes place, and a high maximum is reached. With small inoculations of cocci the addition of more than 0.3 c.c. *N/10* hydrochloric acid results in the death of the organisms within a short time.

With additions of *N/10* soda varying between 0.4 and 1.2 c.c. there seems to be a progressive decrease in the height reached by the maxima, the rate of growth in the early stages is retarded, and the rate of decrease in numbers seems to be retarded. With the addition of 1.6 or 1.8 c.c. *N/10* soda the rate of growth in the early stages is markedly retarded. With the addition of 2.0 c.c. *N/10* soda no growth occurs.

*B. coli* seems to be more sensitive than *S. aureus*, especially to the addition of alkali.

12. More precise experiments with *N/10* hydrochloric acid show that with the addition of increasing amounts of the acid the type of curve gradually changes from a "standard" with one peak to a curve with two peaks, separated by an interval in which the numbers are small.

13. By continuous growth in acid, neutral and alkaline meat extract the capacity of *S. aureus* to multiply when transplanted into media of different



reactions is altered. When transferred into an acid medium all strains show a small primary rise followed, after a fall in the numbers, by a great secondary rise. In the case of the acid acclimatised cocci the secondary rise reached its maximum on the 7th day, in the neutral acclimatised cocci on the 13th day, and in the alkali acclimatised cocci on the 15th day. In the neutral medium there is also a primary and a secondary rise, but the former is much greater than the latter. In the case of the acid acclimatised cocci the primary rise was least in height and duration, and in the case of the alkali acclimatised cocci greatest both in height and duration. In the alkaline medium a primary rise only occurs and subsequently the numbers fall to a very low level. The rise was least in the acid acclimatised cocci and greatest in the alkali acclimatised cocci.

In comparing these experiments with others previously quoted it should be remembered that the organisms have been acclimatised to growth not only in media of different reactions, but also to continuous growth in fluid meat extract medium.

14. *S. aureus* can multiply to a small extent in neutral gelatin solution (8 per cent.). On a medium consisting of gelatin and meat extract the greatest multiplication takes place, much higher figures being obtained than the maxima of growth on gelatin solution and meat extract respectively added together. In agar solution (0.8 per cent.) alone no multiplication takes place, and the cocci quickly die. In a medium consisting of agar and meat extract the maximum reached is lower than in meat extract, but the decline in numbers is slower.

15. When certain quantities of various acids are added to warm meat extract agar precipitates are formed, though little or no precipitate may be produced by lesser or even slightly greater quantities. In some instances no growth occurred in plates poured from those tubes in which a precipitate had formed.

16. The addition of glucose to the extent of 1 per cent. to dilute meat extract results in most cases in *S. aureus* multiplying rapidly during the first day. Subsequently the numbers decline and the culture dies. With increasing quantities of glucose the maximum figure attained diminishes, and the rate of the subsequent fall, at least from the second to the fourth day, increases. Even with a very small quantity of glucose the numbers begin to fall after 24 hours' incubation, instead of rising as they do in cultures without glucose.

If to cultures containing 1 per cent. glucose daily additions of small quantities of concentrated meat extract or of concentrated meat extract with glucose are made oscillations in the numbers occur, but the cultures remain alive and with large additions multiplication may take place. The death of the organisms is not hastened by small daily additions of glucose.

17. The addition at different times of small numbers of the cocci to growing cultures of *S. aureus* has no appreciable influence, but the addition of large numbers exerts a considerable influence.

18. In meat extract cultures of *S. aureus* incubated at 37° C. about 15 per cent. of the living organisms sink to the bottom after each daily shaking. If the tubes are left undisturbed about 25 per cent. sink to the bottom.

19. Meat extract incubated with chloroform for 24 hours at 37° C. and sterilised by boiling seems to be a slightly better medium than fresh meat extract sterilised by boiling or autoclaving immediately after preparation. Pancreas extract is a better medium than meat extract. The multiplication of cocci is greatest in pancreas extract incubated with chloroform for 24 hours at 37° C. and sterilised by boiling.

20. Organisms accidentally contaminating cultures of *S. aureus* may cause, according to their species, a sudden decline or a rapid increase in the number of the cocci.

## REFERENCES.

- BARBER, M. A. (1908). The Rate of Multiplication of *Bacillus coli* at Different Temperatures. *Journ. Infect. Dis.* v. 379-400.
- BUCHANAN, R. E. (1918). Life Phases in Bacterial Cultures. *Journ. Infect. Dis.* xxiii. 109-125.
- BUCHNER, H., LONGARD, K. and RIEDLIN, G. (1887). Ueber die Vermehrungsgeschwindigkeit der Bakterien. *Centralbl. f. Bakteriöl. u. Parasitenk.* II. 1-8.
- CHESNEY, A. M. (1916). The Latent Period in the Growth of Bacteria. *Journ. of Exp. Med.* xxiv. 387-418.
- CHICK, H. (1912). The Bactericidal Properties of Blood Serum. *Journ. of Hygiene*, xii. 414-435.
- COBBETT, L. and MELSOME, W. S. (1896). On Local and General Immunity. *Journ. of Path. and Bact.* III. 39.
- COHEN, B. and CLARK, W. M. (1919). The Growth of Certain Bacteria in Media of Different Hydrogen Ion Concentrations. *Journ. of Bact.* iv. 409-427.
- COPLANS, M. (1910). Influences affecting the growth of Micro-organisms—latency: inhibition: mass action. *Journ. of Path. and Bact.* xiv. 1-27.
- HEHEWERTH, F. H. (1901). Die mikroskopische Zählungsmethode der Bakterien von Alex. Klein und einige Anwendungen derselben. *Arch. f. Hygiene*, xxxix. 321-389.
- KLIGLER, I. J. (1919). Growth Accessory Substances for Pathogenic Bacteria in Animal Tissues. *Journ. of Exp. Med.* xxx. 31.
- LANE-CLAYTON, J. E. (1909). Multiplication of Bacteria and the Influence of Temperature and some other Conditions thereon. *Journ. of Hygiene*, ix. 239-248.
- LEDINGHAM, J. C. G. and PENFOLD, W. J. (1914). Mathematical Analysis of the Lag-phase in Bacterial Growth. *Journ. of Hygiene*, xiv. 242-260.
- MCKENDRICK, A. G. and PAI, M. R. (1911). The Rate of Multiplication of Micro-organisms: a Mathematical Study. *Proc. Roy. Soc. Edin.* xxxi. 649-655.
- MÜLLER, M. (1895). Ueber den Einfluss von Fiebertemperaturen auf die Wachstumsgeschwindigkeit und die Virulenz des Typhus-bacillus. *Zeitschr. f. Hygiene*, xx. 245-280.
- PENFOLD, W. J. (1914). On the Nature of Bacterial Lag. *Journ. of Hygiene*, xiv. 215-241.
- and NORRIS, D. (1912). The Relation of Concentration of Food Supply to the Generation Time of Bacteria. *Journ. of Hygiene*, xii. 527-531.
- RAHN, O. (1906). Ueber den Einfluss der Stoffwechselprodukte auf das Wachstum der Bakterien. *Centralbl. f. Bakteriöl. u. Parasitenk.* II. Abt. xvi. 417-429.
- SALTER, R. C. (1919). Observations on the Rate of Growth of *B. coli*. *Journ. Infect. Dis.* xxiv. 260-284.
- SHEARER, C. (1917). On the Presence of an Accessory Food Factor in the Nasal Secretion. *Lancet*, i. 59.
- SLATOR, A. (1916). The Rate of Growth of Bacteria. *Trans. Chem. Soc.* cix. 2.
- (1917). A Note on the Lag-phase in the Growth of Micro-organisms. *Journ. of Hygiene*, xvi. 100-108.



## THE CONVERSION OF AIR INTO A LETHAL MIXTURE OF GASES BY STORAGE OF TOBACCO AND OTHER VEGETABLE SUBSTANCES.

By ROBERT C. FREDERICK,  
*Civil Assistant to the Professor of Hygiene.*

*(From the Royal Naval Medical School, Royal Naval College, Greenwich.)*

IN analysing some samples of air which had been sent for examination, the writer was struck by the presence of an altogether abnormal quantity of carbon dioxide associated with a very much diminished oxygen content. In view of this excess, the space from which these samples had been obtained was then thoroughly ventilated and after remaining closed for only four days the air was found to contain already 2·27 p.c. of carbon dioxide instead of the normal 0·03 p.c. No immediate explanation of the phenomenon was available and this paper contains a short account of the research made to discover the cause.

The space was an uninhabited and unventilated storeroom. Examination showed that the carbon dioxide could not have gained access from an external source and that it must have been derived from the contents of the store.

Amongst the stores was a considerable quantity of tobacco which was found to be very mouldy. Part was in the form of the whole loose leaf and the remainder was a prepared tobacco mixture contained in cardboard 'war-time' receptacles which could not be as hermetically perfect as the tins which they were temporarily replacing.

Experiments were made under conditions imitating those prevailing in the storeroom to ascertain if, and under what circumstances, tobacco could effect any marked alteration in the composition of the air in which it was stored. For this purpose air sample bottles of 60–70 c.c. capacity were used.

They were thoroughly flushed out with pure air and known quantities of tobacco of varied quality were placed in them. The vaselined stoppers were secured with rubber bands; the subsequent examination showed the necessity for this precaution as a very strong pressure was generated. These bottles were allowed to remain sealed for a period of 18 days (and longer) at temperatures of 12° C. and 37° C., at the end of which time they were opened under mercury and the residual air in them was analysed. The analytical apparatus employed was the Haldane for general air analysis together with the accessories already described for use with the Haldane apparatus for estimation of carbon dioxide only (*J.S.C.I.*, January 31, 1916).

In consequence of the first findings the research was extended to include the action of hay and potatoes.

The experiments and results are summarised below:

Series	Nature of sample employed	Number in series	Temperature at which stored	No. of days stored	Moisture in sample: per cent.	Bone dry weight of sample used: lbs. per cubic foot	Percentage of carbon dioxide found	Percentage of oxygen found	Percentage of carbon dioxide added to air	Percentage of oxygen removed from air	Percentage of total oxygen originally present removed	Combustible gases
A	Export tobacco mixture (prepared with glucose and treated with formaldehyde) very mouldy: wet	1	12° C.	18	54.7	1.27	49.95	0.21	49.92	20.72	99.0	Absent
		2	37° C.	18	54.7	0.76	39.85	0.29	39.82	20.64	98.6	Absent
B	Export tobacco mixture (prepared with glucose and treated with formaldehyde) very mouldy: air dried and friable	1	12° C.	18	9.7	1.29	0.25	20.63	0.22	0.30	1.4	Absent
		2	37° C.	18	9.7	1.54	2.52	18.97	2.49	1.96	9.3	Absent
C	Export tobacco mixture (prepared with glucose and treated with formaldehyde) free from ocular evidence of mould: wet	1	12° C.	18	39.39	1.22	17.47	3.72	17.44	17.21	82.2	Absent
		2	37° C.	18	39.39	1.35	23.73	1.26	23.70	19.67	93.9	Absent
		3	12° C.	41	39.39	1.27	28.05	—	28.02	—	—	—
		4	{ 37° C. 12° C.	{ 18 23	{ 39.39 —	{ 1.21 —	{ 35.61 —	—	35.58	—	—	—
D	Export tobacco mixture (prepared with glucose and treated with formaldehyde) free from ocular evidence of mould: air dried and friable	1	12° C.	18	8.31	1.65	0.05	20.88	0.02	0.05	0.2	Absent
		2	37° C.	18	8.31	1.41	0.53	20.14	0.50	0.79	3.8	Absent
		3	12° C.	32	8.31	1.52	0.08	—	0.05	—	—	—
		4	{ 37° C. 12° C.	{ 18 14	{ 8.31 —	{ 1.58 —	{ 0.60 —	—	0.57	—	—	—
E	Tobacco mixture, pure tobacco only: free from ocular evidence of mould: wet	1	12° C.	18	40.04	1.15	22.68	0.25	22.65	20.68	98.8	Absent
		2	37° C.	18	40.04	1.13	23.28	0.12	23.25	20.81	99.4	Absent
F	Tobacco mixture, pure tobacco only, free from ocular evidence of mould. Bone dry tobacco heated for 3 hours at 98° C. to effect sterilisation: sterile water added, and whole sealed under aseptic conditions: wet	1	12° C.	18	35.11	1.07	4.91	—	4.88	—	—	—
		2	37° C.	18	35.11	1.16	9.52	—	9.49	—	—	—
G	Tobacco mixture, packet of proprietary brand purchased in ordinary retail market. Sterilised and sealed under aseptic conditions as in Series F but more elaborate precautions taken to secure absolute sterility, and sterility confirmed by bacteriological examination commenced at time of analysis. Bone dry tobacco heated for 3 hours on each of three consecutive days at 98° C., moisture in form of 1/250 mercuric chloride added to bottom of bottle where it was absorbed by a small proportion of the tobacco; major proportion of tobacco made wet by subsequent vapourisation, condensation, and absorption of pure moisture	1	15° C.	18	37.0	1.61	3.80	—	3.77	—	—	—
		2	37° C.	18	37.0	1.61	9.79	—	9.76	—	—	—
H	Hay: wet											
I	Potato (peeled): inoculated with pure culture of mould ( <i>Aspergillus</i> ) from tobacco used in Series A	1	12° C.	18	52.75	1.02	21.92	0.25	21.89	20.68	98.8	Absent
		2	37° C.	18	52.75	0.87	24.83	0.26	24.80	20.67	98.8	Absent
J	Mould ( <i>Aspergillus</i> ) inoculated on maltose agar medium		12° C.	40	78.6	0.14	24.21	0.21	24.18	20.72	99.0	Absent
K	Blank control		12° C.	40	—	—	0.30	20.65	0.27	0.28	1.3	Absent
			12° C.	40	—	—	0.03	20.93	Nil	Nil	Nil	Absent



These experiments show that under certain conditions, and after elapse of sufficient time, tobacco stored in an unventilated space converts the air into a mixture of gases entirely incapable of supporting human life and therefore that anyone entering such a space after these conditions had prevailed would be almost instantly killed.

An essential condition for this reaction is that the tobacco be damp; air dried tobacco containing less than 10 p.c. of moisture is not productive of danger even after 32 days. It is to be noted that tobacco in this latter state gives an apparent physical indication of much less than 10 p.c. moisture content.

The degree of conversion is proportionate to time up to a maximum. The presence of mould and micro-organisms accelerates and increases the reaction but it is not the causative agent. The reaction is effected by the tobacco itself and is not due to any material which may be added in the course of manufacture.

The decomposition is increased by heat; the apparent deviation from this in Series A was due to the higher temperature evaporating the water which condensed on the shoulder of the bottle out of contact with the tobacco. Neither carbon monoxide nor any other combustible gas is produced by the reaction.

The same conversion occurs when hay or potatoes are stored in an unventilated space and probably also in the presence of a large number of other vegetable products.

The research has a practical application in showing the necessity for storing damp vegetable products only in spaces where efficient ventilation is assured.

BLACKWATER FEVER.

BY LEONARD S. DUDGEON, C.M.G., C.B.E., F.R.C.P. LOND.,  
*Professor of Pathology in the University of London.*

SYNOPSIS.

	PAGE
Introduction . . . . .	208
Section I. Abnormal changes found in the blood plasma, serum, and red blood corpuscles in blackwater fever. 1. Jaundice. 2. Haemoglobinaemia. 3. Observations on the blood cells. 4. On the serum reaction for syphilis. 5. Parasites: (a) Spirochaetes; (b) Malarial	209
Section II. The tissue changes in blackwater fever. The injection of "malarial melanin" into animals . . . . .	215
Section III. On the extraction of the urine and tissues in blackwater fever for the presence of haemolytic substances . . . . .	223
Section IV. The experimental production of haemoglobinaemia and haemoglobinuria in animals injected with immune sera . . . . .	227
Section V. On quinine in relation to blackwater fever. (a) The administration of quinine previous to and during an attack of blackwater fever. (b) On the haemolytic action of quinine salts in urine and on the excretion of quinine in the urine in malaria and blackwater fever. (c) Quinine administration in rabbits, in which complete occlusion of the ureters had been performed . . . . .	230
Section VI. On the examination of the urine in blackwater fever. 1. Agglutination of red cells in the urine. 2. On the presence of spirochaetes in the urine. 3. On the presence of acetone and diacetic acid. 4. On the presence of haemolytic substances in the urine. 5. On the injection of the urine from cases of blackwater fever into rabbits. 6. On the rapid changes which occur in the urine during an attack of blackwater fever. 7. The colour of the urine . . . . .	236
Section VII. The seasonal incidence of blackwater fever in relation to malaria and climatic conditions . . . . .	240
Summary . . . . .	242
References . . . . .	243

INTRODUCTION.

THE investigations on blackwater fever herein described were made in the Balkans during 1916-17-18, while I was attached to the British Force there, and were completed in my laboratories at St Thomas's Hospital, in 1919 and 1920, after my return from the East. The material, which was unlimited, was derived from patients of *all nationalities* who suffered from blackwater fever and malaria. The study of the disease was made on certain lines and the findings will be recorded in the various sections into which the work is divided.

No attempts will be made to discuss certain of the problems of this interesting disease which have been dealt with by previous workers, many of whom have made observations on cases of blackwater fever year after year in tropical and subtropical countries.



## SECTION I.

ABNORMAL CHANGES FOUND IN THE BLOOD PLASMA, SERUM, AND  
RED BLOOD CORPUSCLES IN BLACKWATER FEVER.

1. Jaundice—bile pigment. 2. Haemoglobinaemia. 3. Observations on the blood cells.
4. On the serum reaction for syphilis. 5. Parasites: (a) *Spirochaetes*; (b) Malarial.

1. **Jaundice.** William Hunter (1908) writing on Jaundice says: "It is characterised by yellowish discoloration of the tissues with bile pigment," but he also refers to a condition known as "Urobilin jaundice" which he regards as a true jaundice, associated with urobilinuria, and due to the formation of urobilin from bilirubin in the intestine. There are others who regard jaundice as a pigmentation of the blood and tissues due to the presence of bile pigments alone, and do not include the pigmentation due to the presence of urobilin. There is little doubt, in my opinion, that if the definition of jaundice were strictly confined to the abnormal discoloration produced by the presence of bile pigments in the blood and tissues, much *greater clinical* accuracy would ensue, as many medical officers in charge of patients employ the term jaundice without any clear definition of its meaning, with the result that any "yellowish appearance" of the skin or conjunctiva is termed jaundice.

Jaundice was observed in 20 of my cases out of a total of 49 in which full records were made. In some of the negative cases a yellowish discoloration of the conjunctiva was noticed, somewhat similar to that which is so well known in patients suffering from pernicious anaemia, but there was no evidence of bile pigment in the blood serum. In the 20 positive cases the yellow discoloration was proved to be due to bile pigment in the blood plasma, while in many instances the jaundice was intense, and in 16 out of the 20 cases a fatal result ensued. In three of the fatal cases, these having died on the fifth and seventh day of the disease, jaundice developed at the onset of blackwater fever, but icterus was absent at autopsy. In some of the negative cases, jaundice was stated to be present on clinical evidence, but bile pigment could not be demonstrated. It will be seen by reference to the post-mortem findings that the bile in the gall bladder was extremely thick and concentrated, especially in the cases which were jaundiced, and such samples of bile were found to be actively haemolytic for human or animal red cells, owing to the presence of a high percentage of bile salts. Concentrated bile occurred, however, apart from jaundice among the cases of blackwater fever.

The method which was adopted for testing the presence of bile pigment in the blood serum or plasma was as follows: Blood serum or plasma mixed with red cells, or free of the latter, was evaporated in a porcelain dish on a water bath to a dry sticky residue. Cold water was then rapidly passed over it, and all excess removed before fuming nitric acid was added with the usual precautions to the dried residue. If bile pigment was present a play of colours developed showing a definite green streak, which varied in intensity according to the amount of bile pigment present. The reaction, however, is quite distinctive and in my experience since Dr A. Wilkin drew attention to the value of rapidly

washing the surface of the dried blood with cold water the test has been found to be accurate and perfectly easy to manipulate<sup>1</sup>.

Albert Plehn (1914), in his classification of blackwater fever into five groups, states that jaundice occurs in four out of the five. Arkwright and Lepper in their report on 16 cases of blackwater fever occurring in the Eastern Mediterranean state that jaundice of some degree was present in 15 cases. In eight instances it was slight; in six it was much more definite; and in one case it was intense. The blood serum gave a marked reaction for bile pigment in the severe case, and "in two or three other cases slight reactions were obtained, probably indicating bile pigment." Bile pigment was not found in the urine in any instance. There is no definite proof that true jaundice occurred in all their cases, as the nature of the pigment in the blood plasma was not determined. It is extremely important to have accurate data as to the nature of the pigment in this disease, and Arkwright and Lepper fully realise this fact as they doubt the wisdom of giving a decided opinion without such positive information. They emphasise that the liver is disordered and swollen in blackwater fever and the bile inspissated.

Phear (1920) has recorded recently some clinical notes on blackwater fever occurring in Macedonia, in which intense jaundice was present in 17 of the 40 fatal cases, while in 13 it was well marked. Throughout the literature of blackwater fever mild to intense jaundice is commonly referred to in the records of cases, but as far as can be ascertained from the data furnished, the diagnosis rests purely on clinical evidence. It is of course unnecessary to adopt any other means in cases of intense jaundice, but in those who show a "lemon tinging" of the conjunctiva an examination of the blood plasma, or serum, should be made so as to ascertain whether bile pigment is the cause of the abnormal pigmentation. The abnormal coloration of the urine in blackwater fever was found to be due in some of my cases to urobilin.

2. **Haemoglobinaemia.** Muir and Dunn (1915*a*) recognise three degrees of haemoglobinaemia, all of which lead to accumulations of iron in the kidneys: (a) a degree too slight to be detected directly (this is probably the case in pernicious anaemia), (b) a degree sufficient to enable the free haemoglobin to be recognised in the plasma; and (c) a degree sufficient to produce also haemoglobinuria. Corresponding to these three degrees of haemoglobinaemia there will be increasing amounts of haemosiderin in the kidneys.

The blood withdrawn from a vein in a case of blackwater fever may show evidence of haemolysis, varying from a deep red coloration of the plasma to a faint tinging. If the blood is withdrawn from a vein into a large test tube ( $6 \times 1$ ) with all reasonable precautions, and immediately sloped, the serum will separate rapidly at  $37^{\circ}\text{C}$ ., or at the temperature met with in the tropics, and the escape of haemoglobin from the red cells will not occur through a technical error, and thus give a false observation. At the end of one hour's incubation at  $37^{\circ}\text{C}$ . the tube of blood is placed in the perpendicular position

<sup>1</sup> The addition of a few drops of water at the end of the reaction will intensify a feeble result.



in the ice safe. The evidence of haemoglobinaemia rapidly disappears from the blood, so that this phenomenon may be overlooked, unless the examination is made at the correct moment. The spectrum obtained is oxy- or met-haemoglobin, or both may be present in the same sample of blood serum.

Arkwright and Lepper (1918) looked for evidence of haemoglobinaemia in ten of their cases, and obtained positive findings in four, while various workers on blackwater fever have shown previously that there is a haemoglobinaemia in this disease. Barratt and Yorke in 1909 brought forward evidence to show that the haemoglobinaemia preceded the haemoglobinuria, while from their investigations on piroplasmosis in dogs they showed that the haemoglobinaemia appears as soon as the blood plasma contains as much haemoglobin as is obtained from an amount of red cells equal to 0.5 per cent. by volume of the plasma. The percentage of haemoglobin in the urine is generally greater than that in the blood plasma.

Muir and McNee (1912) found from experiments conducted on animals with haemolytic immune sera that the amount of haemoglobin passed in the urine is small in relation to the degree of blood destruction. They refer to one instance in which 157 c.c. of dark brown urine were passed in 48 hours, but it contained an amount of haemoglobin corresponding only to 3 or 4 c.c. of blood.

**3. Observations on the blood cells.** Investigations on the total number of red cells per c.mm. and the percentage of haemoglobin were made in many cases of blackwater fever during the period of haemoglobinuria, and in the stage of intense anaemia which may persist when the haemoglobinuria has disappeared, but no estimation on blood volume was attempted. The degree of anaemia in this disease is very considerable, as would be expected, and is often of a rapidly progressive type.

The following case serves as a typical example of this progressive anaemia. Onset of blackwater fever on April 2nd, 1917. On the following day the disease was present with all its characteristic manifestations, and the red cells numbered 2,380,000 per c.mm. On April 4th, the haemoglobinuria was completely arrested, but the red cells numbered 1,900,000 per c.mm., and on the next day the urine again showed haemoglobinuria and the red cells totalled 824,000 per c.mm. Death occurred 48 hours later. At the autopsy the man was jaundiced and most of the internal viscera showed the effects of arterial thrombosis.

In six cases the red cells were counted a few hours previous to the death of the patients with the following results:

1. Case 2	...	...	0,992,000
2. Case 3	...	...	1,330,000
3. Case 12	...	...	1,000,000
4. Case 18	...	...	0,900,000
5. Case 24	...	...	0,824,000
6. Case 25	...	...	1,048,000

Polychromatophilia and punctate basophilia occurred in many instances, and it has been suggested that quinine is contra indicated in such cases, but my observations do not confirm this view. The total number of leucocytes varied from 34,000 to 1600 per c.mm. without any adequate explanation for such marked variations in cases of equal severity.

Hannemann (1918*a*) records the fact that an intense leucocytosis and increase of polymorphs may occur with the rigor, while subsequently there is a leucopenia and an increase of large hyaline cells.

Arkwright and Lepper (1918) refer to the extreme anaemia which may occur after 24 to 48 hours of blackwater fever, and compare the general appearance of the patient to the anaemia following severe haemorrhage. In addition to the severe blood destruction which occurs, haemorrhages into the serous sacs, or externally, may still further drain the system of its normal blood content. Many cases succumb to the severe anaemia which necessarily follows a severe attack of blackwater fever, when the tissues may show the most profound changes including fatty degeneration of the myo-cardium.

Albert Plehn (1914) in his classification of blackwater fever considers that the anaemia on the third, fourth, or fifth day is the most dangerous complication.

Muir and McNee (1912) found from their experiment on animals injected with haemolytic sera that the rate of regeneration of red corpuscles was very rapid. The mean of all their experiments taken together gives the most rapid daily increase to be not much less than 500,000 corpuscles per c.mm.

*Red cell fragility.* Numerous experiments were made on the fragility of the red cells of patients in the acute and convalescent periods of blackwater fever. Whole blood was employed, or washed red cells, or both, but the fragility remained within the limits of normal blood in every instance.

Bijon (1915) found a diminished resistance of the red cells to various strengths of salt solutions in cases of blackwater fever, and believed that he had found an autolysin in the blood.

*Auto- and iso-haemolysis.* The serum and red cells obtained from the same case, and other cases of blackwater fever, were tested for the presence of haemolysins and agglutinins, but negative results occurred in every instance with the patient's own blood (auto), while there was no evidence that sera obtained from cases of blackwater fever had any special affinity for normal human red cells, or that the red cells from cases of blackwater fever were susceptible to the action of fresh human sera.

Fletcher (1913), in the Federated Malay States, tested the serum and red cells from three cases of blackwater fever, but was unable to demonstrate haemolysins or agglutinins for the autogenous red cells, or for other samples of red cells, and was unable to lyse the red cells from the blackwater fever cases with normal sera.

Porak (1918) examined the serum and a watery extract of the organs both fresh, and dried at 56° C., obtained from one case of blackwater fever, on the patient's red cells and healthy cells, but was unable to obtain any evidence of haemolysis. The microscopical changes found in the viscera in this case, however, were not sufficient to warrant a positive result.

In 1909, de Raadt (1917, 2) put forward the suggestion that blackwater fever is due to the formation of certain immune substances which have both a haemolytic and a parasiticidal action, together with an excess of complement in the circulating blood. He believed that the actual onset of blackwater fever depends on the amount of complement present which is free to act with the immune substances formed. Further, he suggested that chill, excessive exercise, and quinine are able to stimulate this increased formation of com-



plement. He conducted some experiments with five healthy Javanese and 15 who were suffering from chronic malaria. In four of the former the haemolytic activity of the serum was unaltered, but increased in one case, while in eight of the latter it was found to be raised from 50 to 100 per cent. In his opinion, it is for this reason that persons suffering from chronic malaria who take quinine when they visit blackwater fever areas are in danger of contracting the disease. The views advanced by de Raadt to explain the causation of blackwater fever however are not capable of experimental proof, while he fails to supply adequate reasons for these various suggestions.

Frieberger and Hartoch consider that intravenous injection of strong hypertonic saline reduces the amount of complement in the blood. De Raadt adopted this method of "treatment" for three Javanese suffering from chronic malaria to whom he gave intravenously 80 c.c. of a 10 per cent. solution of sodium chloride, but failed to observe a reduction in the complement in the blood.

4. **On the serum reaction for syphilis.** It has been suggested that blackwater fever is dependent upon syphilis just as it is known to be the common aetiological factor in paroxysmal haemoglobinuria, although Barratt and Yorke (1909) considered that venereal disease does not predispose to blackwater fever or influence its course when present. Napier (1913) writing in *The Indian Medical Gazette*, in 1913, favoured the view that syphilis was the cause of blackwater fever, further he suggested that the Wassermann reaction should be made in all cases of blackwater fever and if the reaction was positive, salvarsan should be administered. These suggestions made by Napier are exceptionally unpracticable more especially in a disease which occurs under such unfavourable surroundings as blackwater fever, while he brought forward no positive evidence that blackwater fever is dependent on syphilis. The blood serum was tested for syphilis in 25 of my cases, but the results were negative except in the case of a Serb, and an Indian, both of whom were known to have had the disease, while there was no reason to suppose in either instance that syphilis was definitely related to the acute illness. Arkwright and Lepper refer to the examination of the blood in three cases by Capt. M. Douglas, R.A.M.C., with negative results. These examinations were made one and a half, and one month, respectively, after the onset of blackwater fever.

Microscopical examination of the tissues in blackwater fever has failed to reveal any evidence of syphilis. There is, therefore, nothing to suggest that blackwater fever is either related to, or dependent, upon syphilis.

5. **Parasites in the blood.** (a) Spirochaetes, (b) Malarial.

*Spirochaetes.* The blood in cases of blackwater fever was examined at the height of the paroxysm and subsequently, by the following methods, (1) dark ground, (2) Fontana Tribondeau, (3) Levaditi, (4) Giemsa, and (5) Leishman for parasites other than malarial, but without success. The blood was also lysed and then centrifugalised and film preparations made of the débris, but no spirochaetes were seen by this elaborated technique.

*Malarial parasites.* In the hundred cases of blackwater fever which have been investigated a malarial history was obtained in every instance, while

without exception the infection had been contracted during the European war in the Balkans 1915-1918.

Film preparations<sup>1</sup> of the blood in immediate relation to the paroxysm were made in only 40 instances out of the 100 cases examined, and of these in 42 per cent. malarial parasites were demonstrated of which 60 per cent. were malignant rings or crescents. The interval between the attack of blackwater fever and the last attack of malaria is important, but no reliable inference can be drawn from these findings, because a negative history of malaria was of little or no value among our troops in the Balkans, as in many instances men who had never been "sick" and flatly denied the knowledge of any illness resembling malaria, yet, proof positive evidence of this disease was obtained. In 1917, various investigations were made under my direction on the Balkan Fronts for the purpose of detecting malarial carriers and unsuspected cases of malaria, of which certain examples will be quoted: 80 men, who gave no previous history of any illness in the Balkans, were examined for the presence of malarial parasites, with the result that 33.6 per cent. were found to be positive (B.T. 24 and M.T. 3). During the winter of 1916-17, 2836 men were examined on the Struma Front and no less than 835 were found to be positive malarial carriers. The clinical diagnosis of sand fly fever, trench fever, and P.U.O. (pyrexia—unknown origin) gave every chance for error to occur. In one instance with which I am acquainted, the diagnosis of sand fly fever was made in some 20 odd cases by an "enthusiast" who was anxious to show the value of active anti-malarial measures; but the *Plasmodium vivax* was found in the peripheral blood in 80 per cent. of these cases. The small percentage of blood examinations made during the paroxysmal period of blackwater fever is unfortunate because so many competent observers hold that with the blood destruction the parasites disappear also. Stephens (1913) stated "in blackwater fever it is the general experience that parasites when found rapidly disappear" and it is for this reason that the importance of the presence of pigmented leucocytes and melanin has been considered so as to ascertain the relationship between malaria and blackwater fever.

Seyfarth (1918) found that all his cases (Balkans-European War) had had many attacks of malaria, while malarial parasites were present in the blood in nine out of the ten cases, of which eight were malignant malaria and five were fatal. The men also received most insufficient and irregular treatment with quinine for a considerable period. They were underfed and overworked. Stephens, in his article on blackwater fever in Allbutt's *System of Medicine*, showed from an examination of the blood in 95 cases of blackwater fever, that the demonstration of malarial parasites in the blood in this disease depends almost entirely upon the time when the blood examination is made, as illustrated by the following figures:

- (1) Blood examination. Day before onset of haemoglobinuria. Parasites present in 95.6 per cent.
- (2) On the day of the haemoglobinuria 61.9 per cent.
- (3) On the day after the haemoglobinuria 17.1 per cent.

<sup>1</sup> Thick films were employed in the minority of cases.



The results of the figures arrived at by Stephens have led to the view that the severe blood destruction is the cause of this reduced percentage of positive findings, but for this reason one case in my series is worthy of consideration. The haemolysis was intense, the blood serum was deep red, but the blood was shown to be very heavily infected with malignant ring parasites. The patient died within 24 hours from the onset of the acute illness, and the tissues showed a very heavy infection with parasites both intracellular and free. Another case with less pronounced changes in the blood and tissues was also met with. These two cases prove that in spite of very active blood destruction at the time when the blood examination is made, yet a heavy malarial infection of the red cells may persist. It is possible that other factors in addition to the presence of a haemolysin in the blood may explain the reduction of positive findings to which Stephens has drawn attention. Dudgeon (1919) has shown that when a patient receives an intramuscular injection of quinine complete lysis of red cells occurs in the affected muscles, but the parasites may still be seen in large numbers lying among the débris of red cells. Dr Nakayawa confirmed the observations made by Stephens, as he found malarial parasites in 85 per cent. of cases prior to the onset of blackwater fever (M.T. 14, B.T. 2 and Q.M. 1), but only 12·8 per cent. were positive one or two days after the onset. Hatori (1915) examined 26 cases and found parasites in 96·3 per cent. before the attack of blackwater fever commenced<sup>1</sup>.

## SECTION II.

### THE TISSUE CHANGES IN BLACKWATER FEVER.

**The heart.** In 17 cases the condition of the heart muscle was recorded in detail. In the cases which ran an acute course, it was flabby, and there was intense pallor of the muscle substance with sub-pericardial haemorrhages, and the papillary muscles showed well marked mottling. There was dilatation of the heart, especially of the right side.

On microscopical examination, fatty degeneration was present throughout the muscle substance, or in localised areas. The heart was examined for fatty degeneration<sup>2</sup> in 17 cases of blackwater fever, with the result that diffuse fatty degeneration was demonstrated on three occasions, similar to the severe type of fatty degeneration of the heart muscle which has been recorded in acute diphtheritic toxæmia by Dudgeon (1906), while localised areas of fatty degeneration occurred in five cases. It was not possible to demonstrate fatty degeneration of the heart muscle in the nine remaining cases of blackwater fever. In one case in which diffuse fatty degeneration had occurred, and one

<sup>1</sup> Leishman, in 1912, drew attention to certain bodies which he found in the endothelial cells of the visceral blood or lymph vessels, and which he considered might be parasitic (*Chlamydozoa*). He found these bodies in three cases of blackwater fever. See Leishman, W. (1912). Cell-inclusions in the blood in Blackwater Fever. *Journ. Roy. Army Med. Corps*. Vols. XVIII and XIX.

<sup>2</sup> Scharlach R. staining was used throughout for these investigations.

of less severity, the parasites of pernicious malaria were present in the blood stream, while in the remaining cases, melanin was demonstrated in one or more of the viscera. Dudgeon and Clarke (1919) have shown in pernicious malaria that fatty degeneration of the cardiac muscle occurred in 23 out of a total of 45 cases examined, while in five instances diffuse fatty degeneration was met with. It might be argued, therefore, that this degeneration of the cardiac muscle was malarial in origin, quite apart from the toxic action induced on the tissues in blackwater fever. Fatty degeneration of the cardiac muscle in blackwater fever is a pathological process which might be expected to occur because it is a well recognised condition in certain other diseases associated with severe blood destruction, as pernicious anaemia, and streptococcal septicaemia.

Marked perinuclear granularity occurred with and without the presence of fatty degeneration. Sub-pericardial haemorrhages were met with on three occasions. In one case there was diffuse fatty degeneration of the cardiac muscle, and a large intramuscular haemorrhage associated with a wide tract of necrosis; a similar condition occurred in the other viscera in this case owing to widespread vascular changes.

Hannemann (1918*a*) considers that the haemorrhagic state may be due to quinine derivatives cinchonine, chinicine and cinchonicine acting partly locally on the vasomotor system and partly centrally. This view however is obviously incorrect as the haemorrhagic condition in blackwater fever has no relationship to the quinine intake, and, further, in animals which have received experimentally massive doses of quinine, a haemorrhagic state is not induced.

It has been suggested that the changes present in blackwater fever are dependent upon anaphylaxis and Porak<sup>1</sup> has compared blackwater fever to this condition. There is no evidence in my opinion for this suggestion as an explanation of the pathology of this disease, while the tissue changes in blackwater fever are absolutely distinct from what is met with in anaphylaxis. A typical example of death from anaphylaxis is detailed below together with the microscopical findings which certainly confirms my belief in the absence of any similarity between the two conditions. An officer was slightly wounded on the Doiran-Vardar Front, and was given 500 units of anti-tetanic serum hypodermically. Within ten minutes of the injection, he suffered from dyspnoea which became rapidly worse and death occurred within 35 minutes of the inoculation. At the autopsy, sub-pleural haemorrhages were present, and marked congestion of the alveolar walls, and haemorrhages into the lung tissue and bronchi. There was acute emphysema, but no inflammatory reaction. The thymus was enlarged, and showed scattered haemorrhages,

<sup>1</sup> Cleland, in 1909, had suggested that blackwater fever was the resultant condition, the evidence of anaphylaxis to (dead) *Plasmodium* proteid. See Cleland, J. B. (15. x. 1909). Is Blackwater Fever the expression of anaphylaxis to a Malarial *Plasmodium*? *Journ. Tropic. Med. and Hygiene*.



and the spleen also, but there was no evidence of malaria. There were no changes detected in the liver, kidneys, or other tissues. Another case of death from anaphylaxis was similarly investigated. This patient had malaria, but the findings were not sufficient to suggest blackwater fever. The only phenomenon which is common to both conditions is the haemorrhagic state, but this occurs from numerous causes.

No definite relationship was established between the presence and degree of fatty degeneration of the cardiac muscle in these cases of blackwater fever and the rapidity of the disease from the time of onset of symptoms to the death of the patient, although in two out of the three cases which showed marked fatty degeneration death occurred on the third and fifth days respectively from the time of onset of symptoms. It is obvious, however, that this degenerative change of the cardiac muscle in blackwater fever is of the utmost importance more especially when we consider the severe blood destruction and other effects on the tissues which occur. Full attention should be given to these findings by medical officers in charge of cases of blackwater fever<sup>1</sup>.

**The adrenal glands.** Dudgeon and Clarke in the communication referred to elsewhere state that in the examination of the adrenal glands in 35 cases of pernicious malaria the most constant lesion was the reduction of the fat lipid content in the cortical layers. In no less than 30 cases such reduction was noted, while in many instances the loss was considerable.

In blackwater fever similar results have been observed. A reduction of the fat lipid content was one of the commonest pathological changes met with in this disease as in pernicious malaria, while in a few instances no chromaffine was detected in the medulla of the glands by the chromic acid test. It was observed that when the fat lipid in the superficial layers of the adrenal cortex was reduced to a minimum as shown by the fat stains especially Scharlach R., it might be present as fine scattered granules in the deepest layers. Fatty change in the cells of the medulla of both adrenals was met with on one occasion. This patient had recently recovered from active pernicious malaria with numerous ring parasites in the circulating blood, and this was his third attack of blackwater fever within a period of three months. In some cases congestion of blood vessels, haemorrhages, masses of agglutinated red cells in the vessels, and necrosis of gland tissue were recorded.

Paisseau and Lemaire (1916) considered these changes to be of the utmost importance in pernicious malaria. They grouped the pathological findings and the clinical phenomena under the term "acute suprarenal insufficiency."

In one instance of rapidly fatal blackwater fever "colloid-like bodies" which varied considerably in size were numerous in the medulla of the glands.

<sup>1</sup> Castellani refers to the condition of the heart in blackwater fever as follows: "The heart's action should be carefully watched and may require support," but he does not describe the pathological state of the Cardial muscle in this disease. See Castellani, A. and Chalmers, A. (1919). *Manual of Tropical Medicine*. London, Ballière, Tindall and Cox.

**Spleen.** The spleen was enlarged in every case of blackwater fever in this series. In some instances the enlargement was very considerable. In the very acute cases the colour varied from dark red, or chocolate, to almost black, similar to the appearances which the spleen may present in animals which have succumbed to a haemolysin. Recent or chronic perisplenitis was present in all cases. Prominent white Malpighian corpuscles were of common occurrence, while a large, dark chocolate coloured spleen dotted all over with prominent large white Malpighian corpuscles gave a striking appearance which this organ frequently presented in blackwater fever. The microscopical changes were very numerous, but many known to occur in malaria were met with in blackwater fever, which could be explained by the fact that every case had suffered recently or within the last few years from one or more attacks of malaria.

The result of the microscopical examination of the spleen in blackwater fever varied in different cases, according to whether the patient died in the acute stage of the disease, or subsequently from grave anaemia, while the microscopical appearances were not necessarily similar among the cases in either period. The following changes which occurred will be referred to:

Localised patches of necrosis of the splenic stroma; less frequently this condition occurred as a widespread lesion. The sinuses were distended with blood and marked congestion of their walls was observed. Agglutination of red cells in the sinuses occurred and active phagocytosis of red blood corpuscles by the endothelial cells lining the walls or free in the sinuses. The phagocytic reaction when well marked gave a striking appearance to sections of the organ, while evidence of haemolysis in the blood vessels and splenic sinuses still further intensified the picture. Endothelial cell activity was a marked phenomenon in some of the spleens and also the formation of small giant cells. This endothelial cell activity with enlargement of the splenic sinuses developed at the expense of the lymphoid tissue. Thrombosis in the *large* vessels was seen in two cases with infarction of the splenic tissue. The staining of the red cells in the splenic sinuses by a double stain, such as Giemsa or Leishman, showed varying colour effects from the normal red to brown, grey, and shades of blue. This polychromatophilia was very pronounced in some instances. Polymorphs were found to be numerous in the sinuses in a few cases.

**Melanin. Free-iron.** Melanin was present in the sinus cells, or free, and also a true iron-free pigment, while in some cases a very marked free-iron reaction in the splenic stroma occurred. The free-iron reaction was demonstrated in the tissues as small, medium, or large granules, or as a diffuse blue staining of the tissues or individual cells. Free-iron was demonstrated occasionally in the Malpighian corpuscles. Melanin was found to give a free-iron reaction by "unmasking," while in some instances the apparent melanin granules were found to give a free-iron reaction; similar observations were made on the melanin granules in the liver. This, however, was not observed in malaria.

**The Malpighian corpuscles.** It has already been stated that the large size and prominence of these bodies is frequently one of the striking features at



an autopsy in cases of blackwater fever<sup>1</sup>. The enlargement is due to active endothelial cell proliferation with partial necrosis of many of the enlarged cells, while the normal lymphoid tissue content is reduced to a minimum. Large coarse free-iron granules were demonstrated in these bodies occasionally, while melanin was absent or present in very small amount. Dudgeon and Clarke described similar changes in the Malpighian corpuscles in malaria. They state as follows: "Endothelial cell proliferation in the central areas of these bodies is not infrequent, while in some cases these endothelial cells have undergone partial or complete necrosis."

**The gall bladder.** In several cases detailed observations were made on the condition of the gall bladder and of its contents, and the bile passages. The bile in the gall bladder was very thick—in some cases almost solid and of a deep green or orange colour, while the gall bladder in some instances was distended and the bile passages in the liver blocked, with thick tenacious bile. Samples of such bile were boiled to remove bacteria and extraneous substances and the haemolytic action on human red cells was tested, and controlled against a 2 per cent. solution of bile salt in saline.

Dudgeon (1918) has shown that human bile is very variable in its haemolytic action, and while some samples are very inactive, others may be equal to, or even greater than 2 per cent. bile salt in saline. Further he showed that this *haemolytic action* is *intensified* in the presence of *calcium*, but rendered inert by the addition of sufficient blood serum. Concentrated thick bile, which is actively haemolytic, owing to a high percentage of bile salts, is found in the gall bladder in blackwater fever and many other conditions. In several cases of blackwater fever I have found that the concentrated bile was as actively haemolytic, or even more so, than 2 per cent. bile salt. This occurs in obstructive jaundice, although jaundice was not present in each case referred to here at the time of the autopsy, in spite of the presence of inspissated bile in the gall bladder and bile passages. Arkwright and Lepper refer to the marked inspissation of the bile in the gall bladder in their cases of blackwater fever, and compared it to porridge, while in one case with considerable jaundice the bile capillaries were blocked with inspissated bile, and dilation of bile capillaries was also seen.

**The liver.** Numerous changes occur in the liver in blackwater fever, but enlargement and foci of necrosis were met with in every instance. The colour of the liver varied considerably in the large number of cases investigated. In some instances it was dark grey, or light orange, but most frequently a yellowish brown colour. Congestion was of common occurrence, while in several instances haemorrhages beneath the capsule and in the liver tissue occurred, which varied in size from a pin's head to an obvious splash of blood in the liver tissue. The bile capillaries were distended with thick bile such as occurred in the gall bladder.

<sup>1</sup> The prominence of the Malpighian corpuscles may suggest scattered tubercles to the casual observer.

## MICROSCOPICAL EXAMINATION.

The microscopical appearance of the liver in blackwater fever may be relatively slight, but usually marked or intense degenerative and reactionary changes occur similar in many respects to those which accompany certain toxic conditions which act upon the liver cells. Foci of central necrosis of the hepatic cells are among the commonest lesions of blackwater fever, while in some instances wide tracts of liver tissue are necrosed. The haemorrhagic areas which have been referred to may merge into the necrotic tissue, as is seen in an exaggerated form in acute yellow atrophy of the liver. Fatty change in the liver cells occurs in and around these foci of central necrosis, while an intense polymorph reaction may also be met with. The polymorphs are intermixed with necrosed liver cells and the nuclear remnants of the hepatic cells. There may be an intense free-iron reaction in the liver cells, either in the central or peripheral areas, or both, or the liver cells may be filled with a yellow iron-free pigment. The sinuses may be widely dilated and agglutination of red blood corpuscles may be observed. Melanin is found free or in the sinus cells, while in some instances free-iron granules or a diffuse iron staining of the sinus cells together with the melanin particles may occur. Numerous mononuclear cells may be met with in the dilated liver sinuses. The liver cells may contain bile pigment, and the bile passages may be found widely dilated and apparently blocked with bile.

**Pancreas.** The pathological changes met with in the pancreas were common to those found in the tissues which have been already referred to. No instance of fat necrosis or haemorrhagic pancreatitis occurred, although haemorrhages and congestion of blood vessels were of common occurrence. Marked degeneration of the islets of Langerhans was noted on two occasions. The cells were swollen, distorted, and showed nuclear changes, and in one instance sugar was present in the urine, and amounted to 0·16 per cent. in the blood.

**The kidneys.** These organs were often much enlarged, and varied in colour from a deep purple, or deep chocolate, to lighter shades according to the period of the disease in which the fatal result ensued, and whether active blood destruction was in progress. It is obvious, that the post-mortem findings are largely dependent upon the haemolytic factor. Haemorrhages were present in some cases and intense engorgement of the cortex and medulla, while in some instances the pyramids were a deep purple colour and the cortical markings were not recognisable. The microscopical findings varied in different cases, and for the reasons referred to above. When death occurred during the acute stage of the disease the renal tubules were filled with blood serum, granular debris, and agglutinated red cells which were especially well demonstrated in the convoluted tubules. The individual granules in the granular debris may reach a large size and the debris is found in the cells of the tubules and obstructing the lumina, more especially the convoluted and more highly specialised. This granular matter may be shown to contain



traces of free-iron or iron in large quantities. Warrington Yorke (1911) has shown in the haemoglobinaemia due to *piroplasmosis canis*, that large granules occur in the epithelium of the convoluted tubules of the kidneys, and as already stated it is especially in the epithelial cells of the convoluted tubules that the iron granules are found. The epithelial cells of the tubules—more especially the convoluted—may show all stages of degeneration. The cells may be large, swollen, and vacuolated, and the nuclei may have disappeared, or the cells may be lying free in the lumen of the tubules, or may be collected together to form renal casts, but fatty degeneration was not as frequently demonstrated as might be expected. No case was observed in which advanced fatty degeneration of the renal epithelium had occurred. Large tracts of necrosis of the renal tubules were met with in some instances. All grades of congestion of the glomeruli occurred, from a slight to a very intense form, while a similar variation was observed in the blood vessels. Pigment was present in the glomeruli and also in the blood vessels. The endothelial cells of the glomerular tufts were shed in some cases in which death had occurred during the acute stages of the disease, and blood debris was present in the capsules, while the cells covering the capsular walls suffered in a similar manner. Complete blocking of the renal vessels occurred on one occasion, while acute degenerative changes in the muscular coat of the blood vessels was also met with. Acute inflammatory changes in the interstitial tissue did not occur in uncomplicated cases of blackwater fever. The kidneys were examined in a few cases which had suffered from blackwater fever at a previous period, but who died from some other illness. The interval between the attack of blackwater fever and the period when the kidneys were examined varied from a few weeks to some months. In no instance was any microscopical evidence of chronic nephritis found in the examination of the kidneys in these cases. These findings confirm the results obtained by the ordinary methods of urinary examination. Barratt and Warrington Yorke (1909) had arrived previously at a similar conclusion. No albuminuria of renal origin followed the attacks of blackwater fever coming under their notice, and judging from the cases recorded from the literature, blackwater fever is not a cause of nephritis.

**Lungs.** Sub-pleural haemorrhages were met with in a few instances and formed part of the haemorrhagic state which may occur in blackwater fever. Oedema of the lungs was common, while haemorrhages into the alveoli and considerable congestion of the alveolar walls were recorded on many occasions, and also haemorrhages into the lumen of the bronchi. In some instances the pulmonary haemorrhages were widespread and occurred in conjunction with areas of pulmonary collapse and oedema. Thrombosis or embolism of the pulmonary artery produced a fatal termination in two cases. In three instances the alveolar walls were very congested and the red cells contained ring and dot parasites of malignant malaria associated with blackwater fever.

**Thyroid gland.** In a few instances the thyroid gland was examined microscopically. Considerable diminution in the colloid content of the thyroid vesicles was demonstrated, and in one instance active multiplication of the cells lining the vesicles had occurred.

**Skin.** Sections of the skin obtained from various parts of the body were examined microscopically. Pigmentation of the deepest layer of the epidermis was noted on a few occasions, and mononuclear cells containing yellow pigment granules were demonstrated in the corium, but the pigment found in both situations failed to give the free-iron reaction. There was no evidence of acute or perivascular inflammation present.

**Spirochaetes.** The spleen, kidneys, liver, bone marrow, adrenals and brain were examined by the original method of Levaditi, or one of its modifications for spirochaetes, but a negative result was obtained in every instance.

Film preparations of the tissue juices made and stained by the methods of Levaditi, Fontana Tribondeau, and Giemsa yielded the same negative result.

#### ON THE PRESENCE OF FREE-IRON.

It has been shown that the presence of free-iron in the tissues is one of the most characteristic changes in blackwater fever. It is present either as the finest granules or as large coarse blocks of iron and is especially found in the spleen, kidneys and liver. In some instances, cells, in the liver sinuses especially, may be shown to be free from granules, but to stain a diffuse blue colour with the stains for free-iron. Apparent melanin granules in the liver and elsewhere may give a definite free-iron reaction in blackwater fever as if the melanin was "split" during the haemolytic process. The amount of free-iron in the tissues varies very considerably in different cases, and certainly the most intense reaction was found in two cases which had been transfused for severe anaemia.

Muir and McNee believe from their experiments with haemolytic immune sera that the intensity of the iron reaction is not an index of the degree of the blood destruction. They consider, and my results on the tissues of blackwater fever cases confirm this, that the rapidity of blood destruction with resulting haemoglobinaemia and haemoglobinuria is the important factor in the intensity of the iron reaction.

Muir and Dunn (1915*b*) in their experiments on haemolytic anaemia in rabbits found that the excess of iron stored in the organs has been largely absorbed by the time regeneration has occurred and they believe it has been utilised in the blood regeneration. The absorption of iron was most complete from the liver cells.

These investigators (1915*a*) had demonstrated in a previous paper that in acute haemolytic anaemia, attended with destruction of more than half of the blood within three days, nearly all the iron from the destroyed haemoglobin is deposited in the spleen, liver and kidneys. A certain amount of



iron escapes in the urine during the haemoglobinuria, and the amount deposited in the kidneys is roughly proportional to this.

#### THE INJECTION OF "MALARIA MELANIN" INTO ANIMALS.

The spleens obtained from malarial cases were cut into fragments, crushed and minced. The thick juice obtained was found to contain an abundance of melanin which was washed in saline on several occasions, and then injected into rabbits and guinea pigs intraperitoneally. No abnormal results were obtained in any of the inoculated animals. Inoculation experiments were made also with dried splenic powder containing an abundance of melanin, but no ill effects developed in the inoculated animals.

### SECTION III.

#### ON THE EXTRACTION OF THE URINE AND TISSUES IN BLACKWATER FEVER FOR THE PRESENCE OF HAEMOLYTIC SUBSTANCES.

These experiments were undertaken so as to ascertain whether haemolytic substances were present in the tissues, and whether they were excreted in the urine in cases of blackwater fever.

#### TISSUE EXTRACTS.

*Methods employed.* Tissues were minced, and dried with a current of hot air, while alcohol was freely added to assist in the drying process. The dried residue was ground to a fine powder, weighed, and divided into equal portions, to one of which alcohol was added and to the other acetone, and then incubated at 37° C. for 24 hours, but in some cases the extraction with alcohol and acetone was continued for seven days. No advantage was gained by this prolonged method. The extracts were then stored on ice for a few days, filtered, and the filtrate was evaporated to dryness with hot air, and then taken up in 2-5 c.c. of alcohol or acetone, as required. Experiments were conducted also to ascertain whether "haemolytic bodies" could be extracted from the urine in blackwater fever. The following plan which was adhered to throughout except in certain minor details was as follows: In most cases the results were obtained by extracting fresh samples of urine, but in some instances 24 hour specimens were employed. Care was taken to include the deposit which is generally so bulky during the acute stages of blackwater fever. Amounts varying from 50-200 c.c. were employed for these investigations. Each sample was dried in an oven so arranged as to permit a continuous supply of hot air to be driven across the specimen until it was apparently dried, or until it became of a gluey consistency, when further drying produced no greater effect. Absolute alcohol was frequently added and well mixed with the débris during the drying process.

These extracts were diluted with saline and tested with human, sheep or

rabbit red cells. In all experiments a final dilution of 1 in 20 of the red cells in the saline extract was obtained.

The results were recorded after two hours' incubation at 37° C., and one hour on ice.

*Results.* In 11 specimens of urine out of 27 no haemolysis occurred with the alcohol or acetone extracts in saline, while with 16 extracts haemolysis was present in dilutions of 1 in 10 or greater, and on eight occasions it occurred with both the alcohol and acetone extracts. The three most potent extracts acted in dilutions of 1 in 33, 1 in 40, and 1 in 50 respectively and will be referred to subsequently.

A short résumé is given of those cases in which the organ extracts were proved to have *active* haemolytic properties.

*Case 21:* Private P. Admitted to hospital with blackwater fever April 19th, 1918. Died April 24th, 1918. Urine was clear of blood pigment before death. Patient had very marked anaemia, jaundice, and a haemorrhagic eruption. All the tissue changes were very marked on microscopical examination. Patient had taken 80 grains of quinine between the 13th and 18th days of April—that is in the same month as the blackwater fever developed. In 150 c.c. of urine passed at the height of the attack, 0·0135 gm. of quinine alkaloid was extracted, while at the final stage just before death, 0·067 gm. was found in 145 c.c. of urine. The following results were obtained by extracting the urine and kidneys with alcohol and acetone:

	Extract	Haemolytic end point
<i>Urine</i>	Alcohol	1 in 20
	Acetone	1 in 50
<i>Kidney</i>	Alcohol	1 in 200
	Acetone	1 in 300

75 c.c. of urine were used for this examination, while 5 grms. of dried kidney tissue were employed for the alcohol and acetone extractions, and each extract was taken up in 5 c.c. of alcohol and acetone respectively.

*Case 23:* Private E. Admitted to hospital on April 20th, 1918, suffering from blackwater fever, and died two days later. Patient had very severe anaemia, marked jaundice, and suppression of urine. The tissues showed microscopically such changes as are found in the very severe cases of blackwater fever. There is no record of the amount of quinine taken within a week of the onset of the acute symptoms, although it was known that he had had quinine for some time during his residence in the Balkans.

	Extract	Haemolytic end point
<i>Urine</i>	Alcohol	1 in 20
	Acetone	1 in 10
<i>Kidney</i>	Alcohol	1 in 100
	Acetone	1 in 500

50 c.c. of urine were used in these experiments, and the dried residue was taken up in 5 c.c. of alcohol and acetone. 15·2 grms. of dried kidney tissue



were used, of which 7.6 grms. were employed for the alcoholic extract and the same quantity for the acetone. The final residue was taken up in 5 c.c. of alcohol and acetone.

*Case 38:* Private H. was admitted to hospital with blackwater fever on March 24th, 1918, in extremis, and died the following day. He had very severe jaundice with bile pigment in the urine. The abnormal condition of the urine was such as is found in the most severe cases, and the microscopical changes in the tissues were very advanced.

	Extract	Haemolytic end point
<i>Urine</i>	Alcohol	1 in 10
No acetone extract was prepared.		
<i>Kidney</i>	Alcohol	1 in 30
	Acetone	1 in 100

90 c.c. of urine were used for extraction, and the residue was taken up in 6 c.c. of alcohol only. 10 grms. of dried kidneys were used for the extraction; 5 grms. in alcohol and the same amount in acetone, while the final products were taken up in 2 c.c. of alcohol and acetone.

*Case 48:* Gunner S. was admitted to hospital with blackwater fever on April 2nd, 1917, and died seven days later. The urine was free from blood and blood pigments 24 hours before death. There was grave anaemia, marked jaundice, and the microscopical changes in the tissues were very advanced. He had taken 30 grains of quinine during a period of 72 hours before admission to hospital with blackwater fever. The extraction of 200 c.c. of urine showed: 0.010 gm. of quinine alkaloid.

	Extract	Haemolytic end point
<i>Urine</i>	Alcohol	1 in 10
	Acetone	1 in 10
<i>Kidney</i>	Alcohol	1 in 10
	Acetone	1 in 40
<i>Spleen</i>	Alcohol	1 in 10
	Acetone	1 in 200

100 c.c. of urine were used for the drying experiments, and equal portions of the residue were taken up in 10 c.c. of alcohol and acetone. 30 grms. of dried spleen, and 18 grms. of dried kidneys, were divided equally for the extraction with alcohol and acetone. The final extracts were taken up in 10 c.c. of alcohol and acetone respectively.

*Case 31:* Lance-Corporal K. Admitted to hospital in September, 1918, with blackwater fever (second attack). There was slight jaundice, the urinary changes were very severe, but the patient made a complete recovery. No quinine had been given this year previous to the attack of blackwater fever.

	Extract	Haemolytic end point
<i>Urine</i>	Alcohol	1 in 40
	Acetone	1 in 20

75 c.c. of the urine obtained during the acute stages of the disease were employed for the extraction, and 2.25 grms. of the dried residue were taken up in 10 c.c. of alcohol and acetone respectively.

*Case 60:* Private S. Admitted to hospital, April 19th, 1918, with very acute symptoms of blackwater fever, but made a complete recovery.

	Extract	Haemolytic end point
<i>Urine. Fresh sample</i>	Alcohol	1 in 33
	Acetone	1 in 10
<i>24 hours' sample</i>	Alcohol	1 in 25
	Acetone	1 in 10

75 c.c. of fresh urine and a 24 hours' sample were evaporated and dried, and the residue was taken up in 5 c.c. of alcohol and acetone respectively.

**CONTROL CASES.** Many control cases of malaria and other infections were employed for comparison with the findings obtained in blackwater fever. *In no instance*, however, was an active extract obtained. The following cases which give the average findings will be cited briefly.

*Case 1.* Acute streptococcus streptocaemia. 19 grms. of dried liver were extracted with 190 c.c. of absolute alcohol. The final extract was taken up in 10 c.c. of acetone and alcohol. The haemolytic end point was 1 in 12.5.

*Case 2.* Pulmonary tuberculosis. The same quantity of liver was treated in an identical manner as in Case 1. The haemolytic end point was the same.

*Case 3.* Acute nephritis. The kidneys were dried, and the extract was prepared with 10 grms. of dried powder in 100 c.c. of alcohol. The residue from the alcoholic extract was treated with acetone for 24 hours, and then stored in the ice safe and filtered. The haemolytic end point for both the alcohol and the acetone extract was 1 in 30.

*Case 4.* Pernicious anaemia. Patient's colour before death was bright yellow, and his blood plasma was a similar colour. The liver when dried was treated in exactly the same way as in the above experiment. The haemolytic end point for the alcohol and acetone extracts was 1 in 35. 25 c.c. of each extract were evaporated down and the residue was taken up in 5 c.c. of alcohol and acetone, but identical results were obtained in each case.

*Case 5.* Congenital cholaemia. The spleen was removed at operation, dried, and 10 grms. of the dried splenic tissue were treated with alcohol and acetone respectively. The haemolytic end point for each extract was 1 in 10.

*Case 6.* Acute nephritis. An alcoholic extract of the dried kidneys gave a haemolytic end point of 1 in 10.

*Case 7.* Splenomegaly, with severe anaemia. The spleen was removed at operation, and the dried splenic tissue gave a haemolytic end point of 1 in 10 with both the alcohol and acetone extracts.

*Case 8.* Ruptured malarial spleen. An alcoholic extract of the spleen gave a haemolytic end point of 1 in 10. 25 c.c. of this extract were evaporated down, and equal portions of the residue were taken up in 5 c.c. of absolute alcohol and acetone respectively, but an identical haemolytic end point was obtained.

In all these experiments samples of human red cells from cases of malaria, blackwater fever, various infective conditions and normal red cells were employed, but identical results were obtained whichever sample of red cell was used for the experimental enquiry.

The results obtained by *extracting the urine and tissues*, in the manner already indicated, show that a *haemolytic substance or substances are present in the*



*tissues and urine in cases of blackwater fever*, and that similar substances were *not* obtained from the *tissues in other conditions including malaria*. They may be present in both the alcoholic and acetone extracts, although vastly more potent in the acetone, while the alcoholic extracts would appear to be more active when prepared from the urine in cases which recover.

Macleán considers that acetone has an advantage over alcohol in extraction experiments as it dissolves fats and cholesterol, but leaves phosphatides. In practice, however, it is found that acetone is capable of extracting some of the phosphatides, although the amount is very small under the conditions generally employed. Macleán (1918) has considered the results of these experiments on blackwater fever, but is unable to offer an explanation as to the nature of the substance or substances which would induce these results and, therefore, it is most satisfactory to simply record a statement of facts without useless discussion.

*Conclusion.* In blackwater fever haemolytic substances with active properties can be extracted from the tissues, and to a less extent from the urine, and these substances haemolyse human and animal red cells<sup>1</sup>.

#### SECTION IV.

##### THE EXPERIMENTAL PRODUCTION OF HAEMOGLOBINAEMIA AND HAEMOGLOBINURIA IN ANIMALS INJECTED WITH IMMUNE SERA.

Numerous experiments on these lines have been completed by various workers on previous occasions. These observations were made, however, so as to compare rapid blood destruction in animals with the clinical entity termed blackwater fever in the human subject.

Several rabbits were injected intravenously with cats' sera obtained by immunising cats with rabbits' red cells. The methods adopted in each experiment were in the main similar, but certain minor alterations were made for the elucidation of various details which it was hoped would help to throw light on the aetiology of blackwater fever.

It is only necessary to refer to three of the experiments in detail as no further information was obtained beyond what is about to be referred to.

*Experiment 1. Rabbit 35.* This animal received 6.25 c.c. of immune cat serum in five injections during a period of six days. The red cells dropped from 6,000,000 to 1,500,000 per c.mm., the haemoglobin from 100 to 32 per cent., and the leucocytes from 7500 to 4500 per c.mm. Haemoglobinaemia was present. The urine became dark in colour, and gave a spectrum of oxyhaemoglobin. Albumen was present while a few hyaline casts and red cells were seen in a large deposit of débris. When the rabbit's serum and red cells were mixed together auto-agglutination did not occur. Within a period of one week the urine returned to the normal, and the red cells to  $4\frac{1}{2}$  million per c.mm.

<sup>1</sup> Andrew Balfour has suggested that blackwater fever is due to an active haemolysin introduced by a biting insect, which acts on red cells already enfeebled in some way, usually by malaria, sometimes by tick fever. See Balfour, A. (1913). *Journ. Tropic. Med. and Hygiene*, p. 35.

*Experiment 2. Rabbit 36.* This rabbit received 3.5 c.c. of immune cat serum within 72 hours. The red cells dropped from 6,750,000 to 1,400,000 per c.mm. and the haemoglobin from 100 to 30 per cent. during this period. The rabbit's serum showed haemoglobinaemia, and the urine which was dark in colour contained oxyhaemoglobin. There was a large amount of albumen, abundance of debris, and free-iron present in the urine. The animal died at the end of the third day from the onset of the experiment. The urine in the bladder, at the post-mortem examination, was deep brown in colour and contained oxyhaemoglobin, a large amount of albumen, and numerous granular and epithelial casts. The rabbit was bled just before death but no auto-agglutination was present.

*Autopsy.* The spleen was deep red, and very friable. The heart was flabby. The bile in the gall bladder was very thick.

*Microscopically. Kidneys.* The tubules contained coagulated serum, and there was agglutination of red cells and granular debris present. The epithelium of the tubules, more especially of the convoluted, was degenerated, vacuolated, and was being shed. Free-iron was present, and degenerated renal epithelial cells among the debris in the tubules.

*Experiment 3. Rabbit 39.* This animal received 6.75 c.c. of immune cat serum intravenously in three days with the result that the red cells fell from 6,360,000 to 1,170,000 per c.mm., and the haemoglobin from 120 to 29 per cent., while the leucocytes dropped from 7500 to 2420 per c.mm. The urine was dark in colour, and gave a spectrum of oxyhaemoglobin. There was a heavy deposit of albumen, abundance of granular casts, debris, and a few red cells. The rabbit died four days from the onset of the experiment. The urine was collected for 24 hours before death, and from the bladder at the autopsy. Each sample was dried, extracted with alcohol and acetone for eight days at 37° C., filtered, stored in the cold for three days, and the residue was taken up in 5 c.c. of alcohol and acetone. No haemolysis occurred in a dilution greater than 1 in 10 with either extract, and no haemolytic substances were obtained from the dried kidneys. Rabbits' red cells (1 in 20) in saline were employed for these experiments.

*Autopsy.* There was no bile pigment in the blood serum. The fat was bright yellow. The spleen was large, deep purple, and firm. The liver was very large, pale and mottled, while the bile was unusually thick and viscid. The kidneys were very pale. The urine in the bladder was of a bright yellow colour, and contained a heavy trace of albumen, but no casts were seen.

*Microscopical examination. The spleen.* The Malpighian corpuscles showed active endothelial cell proliferation. The free-iron reaction was very marked. The iron was deposited in large clumps in the stroma. Agglutination and active phagocytosis of red cells was present.

The renal changes were similar to those met with in blackwater fever.

*The liver.* Auto-agglutination of red cells was observed, and red cell debris in some of the vessels. Coarse scattered vacuolation of the liver cells existed. Phagocytes containing pigment and iron granules were numerous in the liver sinuses, while diffuse blue staining of these cells also occurred.

### *Discussion of the results attained.*

The microscopical changes produced in the tissue of rabbits by injections of haemolytic immune sera may be widespread and severe in character, or they may be much less intensive.

Muir and McNee (1912) in their experiments on the production of anaemia by a haemolytic serum did not consider that such sera have any important action on the various organs. In one experiment they refer to areas of necrosis in the liver. It would appear, however, from full reference to their paper



that the differences in the findings obtained by these observers and myself are not so great as might seem to be the case.

There are many points of similarity between the experimental production of rapid blood destruction in animals and blackwater fever. The microscopical changes in the kidneys for example in an animal which has received injections of immune sera and in a patient who has died of blackwater fever may be identical. Similar appearances may be observed in the liver, spleen, and in the characteristics of the bile.

The changes observed in the blood and urine correspond in typical examples of blackwater fever and in the experimental haemolytic anaemia.

Potent haemolytic substances have not been obtained, however, by extracting the urine and organs of rabbits which had succumbed to an anti-haemolytic serum such as occurred in blackwater fever.

Experiments on these lines, however, fail to explain the essential points for which such observations are conducted. A man who is suffering from acute malaria, or who has suffered from many attacks of malaria in the past, is suddenly seized with an acute illness which may be of a fulminating character causing rapid death from severe blood destruction and its consequent effect on the tissues, or the disease may be less severe in character and may end in complete recovery, or the acute illness may subside, but result in a very severe anaemia. The cause of this condition which is termed blackwater fever cannot be elucidated by injecting animals with the sera of immunised animals.

The essential factor—the nature of the haemolytic process in man—is unknown, just as the cause of the more chronic haemolytic condition—pernicious anaemia—is unexplained at the present time.

These experiments, however, are of importance in the elucidation of certain problems in blackwater fever which are dependent upon the blood destruction.

The anaemia produced by the unknown toxin of blackwater fever is very rapid and severe (see Section I), while experimentally, as is shown in rabbits 35, 36 and 39, referred to above, a very severe and rapid anaemia was induced. Robert Muir and McNee have shown, and my experiments confirm their results, that as the individual susceptibility of rabbits varies enormously, it is not possible to make any statement as to the degree of resulting anaemia in relation to the dose of the serum injected. They found that the intravenous injection of a haemolytic serum is often followed by toxic effects which may sometimes lead to a rapidly fatal result, but when the initial effects have passed without fatal result, the animals do well and do not lose weight, and the subsequent phenomena depend upon the degree of blood destruction—either an acute and fatal anaemia follows, or an anaemia which is followed by rapid regeneration.

Extra-cellular and intra-cellular haemolysis are both concerned in blood destruction as William Hunter (1901) insisted upon, but Muir and McNee emphasise that the extra-cellular lysis is the more important factor.

The findings in blackwater fever coincide in many respects with the experimental results obtained by Robert Muir and his assistants, and others, on experimental anaemia.

In conclusion, rabbits which were rendered intensely anaemic by injecting immune anti-sera intravenously in the manner indicated, did not develop haemoglobinaemia and haemoglobinuria when treated with various quinine preparations intravenously or intramuscularly.

## SECTION V.

### ON QUININE IN RELATION TO BLACKWATER FEVER.

#### (a) THE ADMINISTRATION OF QUININE PREVIOUS TO AND DURING AN ATTACK OF BLACKWATER FEVER.

Numerous contributions to the subject of quinine treatment for malaria and its relationship to blackwater fever have been made.

Arkwright and Lepper (1918) record that in 15 out of their 16 cases quinine was administered during the period of haemoglobinuria. It was usually given in the form of the bi-hydrochloride in doses of 10 to 15 grains intramuscularly, but some patients received larger doses. It was given also intravenously and orally. These observers state as follows: "We could not detect any evidence that quinine increased or caused a relapse of the haemoglobinuria in our cases."

Hannemann (1918*b*) considers that the haemorrhagic state may be due to quinine derivatives—Cinchonine, Chinicine, and Cinchonidine—which act partly locally on the vasomotor system, and partly centrally.

Stephens (1907) states as follows: "It is the combination of inadequate amounts of quinine and a continuance of slight fever from time to time that seems especially to favour the onset of blackwater fever."

Armand-Delille, Paiseau, and Lemaire (1917) regard a prolonged malarial infection (more than six months) and cold weather as the two predisposing conditions of signal importance in the causation of blackwater fever. All their cases, however, are stated to have received preventative treatment with quinine and in most instances curative quinine treatment also.

Stannus' (1913) experience leads him to believe that in the absence of malaria there is no blackwater fever, while he has never seen this disease among those who had not suffered from a previous attack of malaria treated by quinine.

In 13 cases investigated by me, the amount of quinine taken orally within seven days of the attack of blackwater fever was obtained; seven of these cases recovered and six proved fatal. The largest doses administered during the seven days were 240, 210, and 200 grains, and each case recovered, while the remaining cases received amounts varying from 30 to 140 grains. Every case of blackwater fever among the British troops had received quinine treatment at some time previous to the attack, but either the exact dose is unknown or the relationship between the quinine administration and the onset of the attack. There is no evidence among the records at my disposal



that quinine treatment for malarial infection during the attack of blackwater fever intensified, or prolonged; the haemolytic process.

Matko (1918) has shown that various samples of urine inhibited the haemolytic action of quinine salts in varying degrees. He believed that this inhibitory action was due to acid phosphate of sodium or potassium. In vitro he estimated that the haemolytic effect of 0.04 gm. of sulphate of quinine is neutralised by 0.007825 gm. of acid phosphate of sodium (free from water of crystallisation). The experiments which Matko arranged on these lines led him to believe that blackwater fever is bound up with the phosphatic metabolism.

On this evidence he decided to treat a case of blackwater fever with acid sodium phosphate as follows:

Male, aged 24, had blackwater fever for 16 hours. He was given 200 c.c. of a 2.5 per cent. solution of acid sodium phosphate, and seven hours later the urine was normal, but a sudden relapse occurred for which 200 c.c. of the phosphate solution were again given. Eight hours later the urine was normal, but another relapse occurred. It was decided, therefore, to give an intravenous injection of equal parts of 6 per cent. acid sodium phosphate and sodium chloride. The haemoglobinaemia ceased forthwith and within four hours the urine was normal. The addition of sodium chloride was made on the supposition that it will counteract rigors or severe pyrexia which may be induced by the phosphate.

(b) ON THE HAEMOLYTIC ACTION OF QUININE SALTS IN URINE AND ON THE EXCRETION OF QUININE IN THE URINE IN MALARIA AND BLACKWATER FEVER.

The samples of urine figured in Table A were taken from the nephritis clinique under Prof. Hugh Maclean, at St Thomas's Hospital. The analysis of the albumen content in each sample of urine was made in his Department and is illustrated in the last column in Table A. It will be readily appreciated from these results that the albumen content has no relationship to the

Table A.

No. of case	Urine l c.c.	Urine containing 1% Quinine Bi-hydrochloride				Albumen
		0.5 c.c.*	0.25 c.c.*	0.2 c.c.*	0.1 c.c.*	
1	—	C.	I.C.H.	I.C.H.	—	—
2	—	C.	I.C.H.	M.	—	—
3	—	I.C.H.	—	—	—	Trace
4	—	C.	I.C.H.	—	—	Trace
5	—	C.	I.C.H.	M.	—	+
6	—	C.	C.	I.C.H.	—	+++
8	—	I.C.H.	—	—	—	Trace
9	—	C.	C.	I.C.H.	—	Trace
10	—	C.	I.C.H.	M.	—	Trace
11	—	C.	M.	—	—	—
12	—	I.C.H.	Trace	—	—	Faint trace

\* In each experiment normal saline was added up to 1 c.c. (Tables A and B).

0.05 c.c. of a solid suspension of washed human red cells was added to every tube.

All tubes were incubated for one hour at 37° C. and the results were read after 30 minutes at room temperature.

*Abbreviations employed.*

C. Complete haemolysis. I.C.H. Incomplete haemolysis. M. Marked haemolysis (a definite deposit of red cells left). Trace. Tinging of solution with haemoglobin. — No haemolysis.

haemolytic activity of urines treated with quinine in the manner about to be indicated. Each sample which was freshly passed was cleared by centrifugalisation and then bi-hydrochloride of quinine was added to make a 1 per cent. solution. Control tests were made with a 1 per cent. solution of quinine bi-hydrochloride in saline. The untreated urines (1 c.c.) had no haemolytic action on a 5 per cent. suspension of washed human red cells in saline, but those to which quinine was added gave varying results, which will be referred to in detail. In several instances the effect of the addition of the urine was inhibitory, while in some of the cases the inhibition was very marked. No increased haemolytic activity was observed. The results expressed in Table B

Table B.

To compare the action of a 1 per cent. solution of bi-hydrochloride of quinine in urine on a 5 per cent. and a 2 per cent. suspension of washed human red cells in saline.

*Amount of Bi-hydrochloride of Quinine in Urine in 1 c.c.*

No. of case	Red cell suspension %	0.2 c.c.	0.15 c.c.	0.1 c.c.	0.5 c.c.
1	5	I.C.H.	M.	—	—
	2	C.	M.	—	—
3	5	—	—	—	—
	2	—	—	—	—
4	5	—	—	—	—
	2	I.C.H.	M.	—	—
8	5	—	—	—	—
	2	—	—	—	—
10	5	M.	Faint trace	—	—
	2	C.	C.	C.	Trace
11	5	—	—	—	—
	2	C.	Trace	—	—
12	5	—	—	—	—
	2	—	—	—	—

*Controlled Test with 1 per cent. of same Quinine Preparation in Saline.*

A	5	I.C.H.	M.	—	—
B	2	C.	C.	C.	Trace

were derived from the same samples of urine treated with quinine, but the comparative haemolytic action on a 5 per cent. and a 2 per cent. suspension of washed human red cells was determined. The chief fact derived from these experiments is that most samples of urine to which 1 per cent. quinine bi-hydrochloride has been added are much less actively haemolytic on a 5 per cent. or 2 per cent. suspension of red cells than saline containing the same quantity of bi-hydrochloride of quinine, but in one instance (case 10) no inhibition occurred<sup>1</sup>.

In Table C the results of the analyses of ten samples of urine from malarial patients are recorded which were made by Captain C. E. C. Ferrey, O.B.E.,

<sup>1</sup> Similar experiments were made with other samples of urine, but the results were in agreement.



in the Central Laboratory, Salonika. Each patient was taking 30 grains of the sulphate or bi-hydrochloride of quinine three times daily. The total quantity of urine passed during 24 hours is recorded, together with the total amount of quinine alkaloid extracted, and the resulting percentage of the alkaloid. These results were obtained from various cases of malaria which were under investigation and they serve to illustrate the variable quantities of urine passed by malarial patients in 24 hours, and the wide variation in the amount of quinine which is extracted. Many more observations were made on the excretion of quinine in the urine of malarial patients, but the results recorded in Table C are sufficient for the purpose. In the Balkans,

Table C.

The quinine analysis of the urine in ten cases of malaria.

No. of case	Total volume of urine passed in 24 hours c.c.	Total amount of quinine alkaloid extracted gramme	Quinine alkaloid in urine %
1	575	0.24	0.04
2	2240	0.25	0.01
3	3110	0.12	0.003
4	2130	0.21	0.009
5	2490	0.28	0.01
6	2900	0.38	0.01
7	2105	0.36	0.017
8	1530	0.64	0.04
9	1850	0.16	0.008
10	500	0.29	0.06

the extremes of temperature met with have an important bearing on the intake of fluids and the amount which is excreted by the kidneys, and, therefore, on the quinine concentration in the urine. The percentage of quinine excreted in the urine in at least three out of the ten cases is sufficient to excite haemolysis of human or other red cells. Such percentages as 0.06 and 0.04 fall within the limit of quinine concentration capable of exciting haemolysis, when the bi-hydrochloride is dissolved in saline, or even in urine as shown in Table B. Further, we must allow for the fact that quinine bi-hydrochloride contains only 81.6 per cent. of alkaloid. Of course it is possible that quinine is present in greater concentration in the urinary tract at some period of its excretion than the above records show. Marshaux's observation, quoted by Stephens, that "during the period of haemoglobinuria quinine cannot be detected in the urine, but reappears on its cessation," is now known to be incorrect. In Table D the results of the analytical investigations of the urine in cases of blackwater fever are recorded, and in all instances, but one, the observations were made at the height of the haemoglobinuria. In each case, the whole sample of urine passed during 24 hours was collected, thoroughly mixed, and 100-200 c.c. were set aside for the quinine estimation. The percentage of quinine alkaloid obtained in each case is recorded together with the amount of quinine intake

when it was known. It would be incorrect to make an absolute comparison between the results recorded in Tables C and D, but the evidence at hand shows that the percentage of quinine alkaloid in the urine in cases of black-

Table D.

*Amount of Quinine Alkaloid recovered from the Urine in  
ten cases of Blackwater Fever.*

The results of the examination of the urine in ten cases are recorded, and are expressed in a tabular form, as follows :

No. of case	Amount of urine employed in estimation c.c.	Quinine alkaloid in sample of urine %	Amount of quinine administered immediately preceding or during the acute illness
18	125	0.029	90 grains in 3 days
21	150	0.009	600 grains in 3 weeks immediately preceding attack
22*	145	0.046	89 grains intravenously and 30 grains orally during attack
24	100	0.005	40 grains in the 24 hours previous to the estimation
26	125	0.029	No record
30	125	0.006	75 grains in last 3 days
32	125	0.009	No record
37	200	0.017	No record
48	200	0.005	30 grains 3 days previously
50	170	0.0015	No record
52	180	0.004	No record

\* This sample was obtained immediately the urine was free from haemoglobin.

water fever during the height of the disease and in malaria may approximate, and that the quinine concentration may be sufficient to excite haemolysis of human red cells.

Nierenstein (1919) described his experimental results with the urine obtained from cases of blackwater fever. He isolated a substance—haemoquinic acid—a disintegration product of quinine, which has pronounced haemolytic properties on human and sheep red cells. The urine from 13 cases of blackwater fever was examined in which this substance was obtained on twelve occasions. Nierenstein suggests that there is a possible relationship between the formation of haemoquinic acid in the organism and the production of blackwater fever, which could be described as a specific quinine “idiosyncrasy” since apparently in blackwater fever haemoquinic acid is produced in larger quantities than in cases of malaria with rigors, the ratio being 578:1. He showed in this paper that in 42,000 c.c. of mixed urine from malarial patients the amount of haemoquinic acid obtained was 0.3 gm., while in 21,000 c.c. of urine from cases with malarial rigors it amounted to 0.18 gm., and in 8400 c.c. of urine from cases without rigors none was found. Contrast the results obtained by Nierenstein in three out of 13 blackwater fever cases where he found (1) 5090 c.c. with 0.36 gm., (2) 1840 c.c. with 0.28 gm., and (3) 3370 c.c. with 0.32 gm. No records however are given of the haemolytic action of haemoquinic acid on human or sheep red cells to which reference is made. Further, it must be fully recognised that the haemolytic action of quinine preparations and of other substances in vitro, cannot be taken as an index of haemolysis by the same substances when present in the blood and tissues of man and animals.



(c) QUININE ADMINISTRATION IN RABBITS, IN WHICH COMPLETE OCCLUSION OF THE URETERS HAD BEEN PERFORMED.

These experiments were conducted for the purpose of ascertaining whether haemoglobinuria, or a condition similar to blackwater fever, could be induced in rabbits in which the ureters were completely occluded, if quinine was administered intravenously, or intramuscularly, just previous to the ligation, or subsequently. It is well known that quinine is excreted mainly in the urine and to a much less extent by the intestinal tract and by the sweat glands. Hartmann and Zila (1918) consider that 40 per cent. of administered quinine is excreted in the urine and faeces, the rest is not deposited in the organs and must, therefore, be destroyed. After intravenous injection, they state that the amount in the blood sinks rapidly at first and afterwards more slowly, while there is no quinine in the blood eight hours after the injection of 0.5 gm. Oral administration, on the other hand, gives a more constant quinine level which is retained for about 24 hours. It was thought possible if sudden suppression of urine was induced in rabbits, and at the same time quinine was administered freely, that in the few days allotted for such experiments haemolytic phenomena might be induced, owing to storage of quinine in the blood and tissues. Five rabbits were selected for these experiments, numbered *a*, *b*, *c*, *d*, and *e*. The abdomen was opened in each case, and both ureters were tied high up near the renal pelvis. Death occurred from the third to the seventh day. Rabbits *a* and *c* were used for control purposes. In these animals the ureters were tied, but quinine was *not* administered, while in the case of rabbits *d* and *e* quinine injections were given to the animals intravenously after ligation of the ureters, and with rabbit *b*, before the operation it was injected intramuscularly, and subsequently intravenously. The concentrations of the quinine solutions employed were 1 per cent. and 5 per cent. of the bi-hydrochloride in saline, which are known to actively haemolyse animal red cells in vitro. Quinine administration in man is at times controlled by giving known quantities of the alkaloid per body weight for the treatment of malaria. This method in my opinion is a mistake because two individuals of vastly different weights do not necessarily show any difference in their quinine tolerance. The rabbits employed in these experiments received an excess of quinine on the body weight ratio.

*Rabbit b.* This rabbit was weighed daily for three weeks, and was injected in the various muscles with 1 c.c. of a 1 per cent. solution of bi-hydrochloride of quinine in saline. At the completion of three weeks' quinine treatment, both ureters were tied, and two injections of 1 c.c. of the 1 per cent. solution of quinine were given intravenously. The animal died two days later. The blood was examined during life, but there was no haemoglobinaemia.

*Post-mortem examination.* Both kidneys were very large, very pale and soft, and showed distension of the ureters proximal to the ligatures, and of the pelvis and calices of each kidney. The fluid obtained from the distended ureters was a reddish brown colour and showed masses of red cells with some slight degree of haemolysis, and contained numerous epithelial cells. Professor Hugh Maclean extracted 0.0035 gm. of alkaloid quinine from

the concentrated urine. There was no jaundice, and the spleen and liver showed no resemblance to the naked eye changes met with in blackwater fever.

*Microscopically.* Kidneys. There was no free-iron present. Traces of fine fat droplets were seen in the epithelial cells of the convoluted tubules. There was no resemblance, however, in either kidney to the lesions found in blackwater fever.

*Rabbits d and e.* The ureters of both these rabbits were tied, and then two intravenous injections of 1 c.c. of a 1 per cent. solution of the bi-hydrochloride in saline were given on the day the ureters were occluded, while four subsequent injections, at daily intervals, of 1 c.c. of a 5 per cent. solution of the bi-hydrochloride were employed.

*The blood.* This was examined on several occasions but there was no haemoglobinaemia, and no bile pigment in the plasma.

*Post-mortem examination.* One rabbit died in five days and the other in seven days, but the changes found were similar in each instance. The kidneys were very enlarged, very pale, and soft, and contained turbid fluid which distended the pelvis of each kidney and the calices. The deposit in each instance contained hyaline casts and red blood cells and some blood débris, but the urine did not resemble that excreted in blackwater fever. The slight degree of haemolysis which did occur could be explained by the concentration of the urine in the distended pelvis of each kidney. The urine from rabbit *d*, cleared by centrifugalisation and diluted with saline 1 in 5, showed marked haemolysis in two hours at 37° C. on a 2.5 per cent. suspension of washed rabbit red cells in saline, but none with a 5 per cent. suspension. The spleens were not enlarged or altered in colour and there were no microscopical changes in the tissues, such as occur in blackwater fever.

The control rabbits, *a* and *c*, were employed so as to compare the effects on the kidneys and urine of complete occlusion of the ureters apart from quinine administration, but the results were similar. The débris from the pelvis of each kidney showed some red cells, together with epithelial cells, but no casts. There were no changes, however, in the kidneys microscopically as met with in blackwater fever.

*Conclusion.* The effect of complete occlusion of both ureters in rabbits while quinine is being given or has been given intravenously or intramuscularly does not induce a condition resembling blackwater fever, in any respects.

## SECTION VI.

### ON THE EXAMINATION OF THE URINE IN BLACKWATER FEVER.

1. *Agglutination of red cells in the urine in blackwater fever.* A study of the microscopical changes in the kidneys in blackwater fever may reveal the presence of "clumped red cells" lying in the lumen of the convoluted and other tubules. The agglutinated red cells may present a striking picture in sections of the kidneys obtained in the acute stages of this disease. For this reason the urine was examined for the presence of agglutinated red cells, which were demonstrated on four occasions. In many instances several samples of urine were examined from the same case. The appearances of the clumped red cells mixed with dark blood stained urine were quite characteristic, although as far as I am aware they have not been referred to previously, while these agglutinated red cells in the urine were found to be very abnormal.



In each case an identical result was obtained. The clumped red cells were well washed in saline, and then treated with powerful haemolytic agents such as distilled water and bile salts alone or with the addition of calcium. It was found that these red cells showed great resistance to these reagents, and further calcium did *not* intensify the haemolytic action of bile salts, but induced an opposite effect.

These experiments were carefully controlled because of the exceptional findings, in fact, owing to the increased resistance to haemolysis it was doubted at first whether these particles were really clumped red cells, but the action of bile salts furnished the necessary proof. It would appear, therefore, that red cells which had resisted haemolysis were excreted in the urine, but were protected from haemolytic agents probably owing to the formation of a coat of some colloidal substance.

Dudgeon (1918) has shown, as already stated, that the addition of calcium to bile salts increases their haemolytic action, but in the case of the agglutinated red cells found in the urine in blackwater fever, calcium has an exactly opposite effect.

Case	Type of case	Examination of the clumped red cells in the urine
1	Rapidly fatal case with haemorrhages.	Did not haemolyse in distilled water. Haemolysed with bile salts, but not with the addition of calcium.
23	Rapidly fatal case with haemorrhages.	The clumps of red cells showed marked haemolysis in bile salts, but there was no increased haemolytic action on the addition of calcium.
51	Rapidly fatal case with haemorrhages.	2 % bile salt induced slight haemolysis at 37° C., but none with distilled water.
55	Recovered.	1 % bile salt induced marked haemolysis in 4 hours at 37° C., but none in the presence of calcium.

2. *On the presence of spirochaetes in the urine.* Film preparations were made of the centrifuged deposit and stained by the original Levaditi method, or that of Fontana Tribondeau, or in those cases in which a very abundant deposit was obtained sections were cut of it after fixation in Kaiserlin and staining the sections by one of the silver methods. No spirochaetes were found, however, by any of the methods employed in the 35 samples of urine investigated. This result corresponds to the negative findings referred to elsewhere when the various tissues were examined from cases of blackwater fever. The absence of spirochaetes from the urinary deposit in every case is also of interest in view of the statements made that the urine in health among the soldiers in the Allied Forces contained spirochaetes apart from any true spirochaetal infection. Fiessinger (1918) considered from his investigations that spirochaetes are found in the urine in about 1 in 45 of normal persons.

In numerous cases, the fresh urine obtained during the height of the attack of blackwater fever was injected intraperitoneally into guinea pigs, while 24 hours' samples of urine were sterilised with ether according to the method of Fildes and Rajchman, before they were used for animal inoculation. No positive results were obtained, however, with any of the samples which were used for inoculation purposes. In one instance a guinea pig which had

been injected with the urine from a case of blackwater fever showed spirochaetes in the liver. It was the only animal in which spirochaetes were found in the whole series, but unfortunately no transference experiments were made as the organisms were not found until sections had been prepared. In 1917, Major A. L. Urquhart, O.B.E., R.A.M.C., and I carried out certain investigations on cases of sand fly fever in the Struma valley. In these experiments, blood obtained by vein puncture during the height of an attack of sand fly fever, was injected into guinea pigs, and after a definite interval the livers of these pigs were injected into other pigs. Spirochaetes were found in the liver tissue of three pigs which were not related to our experiments. These facts are referred to here because they show that spirochaetes are found in the liver of guinea pigs in conditions of apparent health, and are not necessarily pathogenic. The guinea pigs were obtained from Egypt and Malta and the health of these animals on the whole was about the average.

3. *On the presence of acetone and diacetic acid.* The urine from 43 cases of blackwater fever was examined for the presence of acetone and diacetic acid at the height of the haemoglobinuria. On 37 occasions the acetone reaction was negative, and positive in six cases, while the reaction for diacetic acid was negative in 39 out of the 43 samples examined. The tests were frequently applied to the fresh samples and to the 24 hours' specimens. In most instances, the examinations were made by Capt. C. E. C. Ferrey, O.B.E., R.A.M.C., Analytical Chemist to the Army in the Balkans. The large number of negative results for acetone and diacetic acid is of importance. Burkitt (1915) states that the urine is highly acid in blackwater fever and contains acetone, and recommended calcium chloride and potassium or sodium bicarbonate for the treatment of this disease. While at a later date gum arabic 6 per cent. in bicarbonate of sodium has been regarded of the utmost value.

4. *On the presence of haemolytic substances in the urine.* Urine obtained from many cases of blackwater fever at the onset of the attack was tested on the autogenous and other samples of human red cells other than those from cases of blackwater fever, and sheep red cells. 24 hours' samples of urine were tested after centrifugalisation, and sterilisation with chloroform at 45° C., on 5 per cent. washed red cells with and without the addition of human or guinea pig complement employed at various dilutions, but in no instance was haemolysis induced. Weak solutions of quinine in saline were added to the sterilised urine and red cells, but no haemolysis occurred.

5. *On the injection of the urine from cases of blackwater fever into rabbits.* The urine was sterilised at 45° C. with chloroform, previous to the inoculations.

1. *Rabbit 1.* Injected intravenously.

Date	Amount c.c.	Weight grms.
30. vi. 18	0.75	1.163
1. vii. 18	1	—
2. vii. 18	2	—
5. vii. 18	1	—
8. vii. 18	2	—
12. vii. 18	2	—
17. vii. 18	—	1.095

The rabbit showed no ill effects from the inoculations and presented no abnormal blood changes.



2. *Rabbit 2.* This animal was injected intraperitoneally with the urine from blackwater fever cases sterilised as above.

Date	Amount c.c.	Weight kilos
5. vii. 18	2	1.150
8. vii. 18	2	—
12. vii. 18	2	—
17. vii. 18	—	1.115

This animal showed no ill effects from the inoculations.

The urine employed for these injections into rabbits 1 and 2 was mixed samples obtained from cases of blackwater fever during the acute stages of the disease.

6. *On the rapid changes which occur in the urine during an attack of blackwater fever.* Very rapid changes occur in the urine in blackwater fever as the disease responds to treatment. Albumen oxy- or met-haemoglobin, and débris which so suddenly appear at the onset of the disease and are followed by an abundance of renal cells and casts, as rapidly disappear, and the urine returns to the normal. The whole process may be completed within a few days. The rapid improvement found in the urine is in accordance with the results of the microscopical examination of the kidneys after blackwater fever, more especially in comparison with the active changes present in cases which have succumbed during the acute stages of the disease.

The changes which the urine undergoes can be divided into three stages as follows:

1st stage. Albuminuria, haemoglobinuria and abundance of débris.

2nd „ Abundance of renal cells and casts.

3rd „ The return to the normal.

The records of three cases referred to here are taken from data supplied to me by Capt. J. F. Taylor, R.A.M.C., Pathologist to 52nd General Hospital in the Balkans, to whom I am most grateful.

#### Case 1.

	Date	Time hrs.	Amount c c.	Analysis
(1)	7. iii. 18	9.00	150	Dark lager. Albumen. Oxyhaemoglobin débris abundant
(2)	„	11.15	130	„ „ „
(3)	„	14.5	220	Dark sherry colour, otherwise same
(4)	„	18.30	230	Pale sherry. Albumen and oxyhaemoglobin as before. Renal cells and casts abundant
(5)	8. iii. 18	1.00	120	Pale yellow, no albumen, no spectrum, renal casts present
(6)	„	11.00	160	„ „ „
(7)	„	19.15	140	Pale yellow, no albumen, no spectrum, no cells, no casts

#### Case 2. *Pte B.*

23. iii. 18	10.00–18.00	320	Dark porter. Albumen. Oxyhaemoglobin. Renal cells. Granular débris. Some granular casts
24. iii. 18	11.00	200	„ „ „
„	14.00	527	„ „ „
25. iii. 18	—	—	Pink. Albumen trace. No spectrum. Few granular casts and epithelial cells
26. iii. 18	—	—	Yellow. Albumen faint trace. Large number of cells and granular casts
27. iii. 18	—	—	Yellow. No albumen. No casts. Few renal cells
29. iii. 18	—	—	Yellow. Urine normal except for few renal cells

*Case 3. Rfl. J.*

Date	Time hrs.	Amount c.c.	Analysis
11. xii. 17	8.00	—	Oxyhaemoglobin. Dark red with black granular deposit. Albumen
„	22.00	—	No absorption bands present
12. xii. 17	—	—	Almost normal urine to look at. Albumen. Degenerated epithelial cells and few granular and epithelial casts. Débris
12. xii. 17	12.00	—	Trace of albumen and granular casts. Some débris
13. xii. 17	—	—	Trace of albumen. Granular casts
19. xii. 17	—	—	Urine normal

Many other cases could be cited which show similar results, but no advantage would be gained by useless repetition.

Red cells which are present in relatively small numbers rapidly disappear from the urine in blackwater fever as the acute stages of the disease subside, but by accurate examinations of centrifugalised samples of urine red cells are found to occur more frequently in the acute stages of the disease than is generally believed or taught. Warrington Yorke, in 1911, refers to the presence of red cells generally in small numbers in the urine during the period of haemoglobinuria.

7. *The colour of the urine in blackwater fever.* Abnormal variations in the colour of the urine in this disease occur in a more exaggerated degree than in any other known condition in man. The urine may be black or various shades of brown, or deep red in colour. The spectra of oxyhaemoglobin or methaemoglobin are obtained, or both spectra occur in the same sample, one or other in greater proportion.

Burkitt (1919) considers that the "blood red" urine of blackwater fever which gives a spectrum of oxyhaemoglobin only occurs in the worst cases. Barratt and Yorke (1909) showed that only a fraction of haemoglobin injected into the circulation appears in the urine, the rest is employed by the tissues. They also showed that a brownish soluble pigment appears in the urine as the haemoglobin is destroyed which does not give the characteristic spectroscopic bands.

## SECTION VII.

THE SEASONAL INCIDENCE OF BLACKWATER FEVER IN RELATION  
TO MALARIA AND CLIMATIC CONDITIONS.

In 53 per cent. of the 100 cases of blackwater fever which form the subject matter of the present communication, the disease occurred during the months of March and April, while during June and July only one case was met with.

*Climatic conditions.* These records are based on the observations made by the Meteorological Dept. (R.E.) G.H.Q., B.S.F., in 1917 and 1918. The findings for 1916 are omitted for special reasons. In 1917, the absolute maximum temperature for February and the first week in March was at the 60° level, while the mean temperature varied between 42° and 46°. During the second week in March the temperature *began* its upward rise for the summer months. The absolute maximum varied between 64° and 78° and the mean daily temperature from 48°–60°. In April, the absolute maximum fell between 58° and 80°, and the mean daily between 53° and 60°. The wet bulb during the whole of this period was below 65. This brief reference to temperature refers to the observations made in Salonika, but the records taken on the Doiran front were at a slightly lower level, while on the Struma



front the temperatures during the periods referred to were both slightly below and above the Salonika records. In 1918, the first three weeks of February showed an absolute maximum between  $60^{\circ}$  and  $67^{\circ}$ , and a mean daily which varied between  $42^{\circ}$  and  $48^{\circ}$ , while during the last week in February and the first fortnight in March all temperatures were at a still lower level. The absolute maximum records for the rest of March and April varied between  $70^{\circ}$  and  $80^{\circ}$  and the mean daily between  $47^{\circ}$  and  $62^{\circ}$ . Both the absolute maximum and the mean daily temperature on the Doiran-Vardar sector and the Struma front were at a slightly lower level than is recorded for Salonika. The wet bulb records at these periods were below  $65^{\circ}$ . It will be seen from the brief reference to the climatic conditions that the temperature was commencing to rise in March from the low winter records, while at the end of this month and during April the rise was very definite, and it is during this period between the extreme cold of winter and the heat of summer that blackwater fever was most common in the Balkans. As soon as the extreme heat of June and July occurred blackwater fever became a negligible factor.

*Malaria.* The total deaths from malaria during the months of February, March, and April 1917 were four in number, and for 1918 during the same periods they amounted to twelve. A larger number of deaths occurred in one day in the summer months than in three months in this season of the year. These results clearly show that the lowest death rate from malaria is the period when blackwater fever is at its height. The figures about to be quoted refer to the official returns, and to the findings of the various laboratories attached to the British Force, but the official returns include the cases diagnosed on clinical evidence in addition to those in whom the parasites were found. Both records show, however, that at this season of the year malaria is very largely of the benign tertian type, while further it is figured that the rise in the malarial incidence has definitely begun. The laboratory findings, illustrated in the second and third transverse line of figures, clearly show that blackwater fever occurred at the period of each year when malignant malaria was at its lowest limit. In one week in the late summer months as many as 200–300 cases of malignant malaria would occur.

*Malaria.* 1917.

February	262	217	253	381	—	Total admissions
	89	138	162	214	—	Benign tertian
	14	7	6	6	—	Malignant malaria
March	378	459	470	551	675	Total admissions
	164	213	246	249	261	Benign tertian
	4	25	5	4	4	Malignant malaria
April	568	487	561	617	—	Total admissions
	202	244	281	270	—	Benign tertian
	1	2	0	0	—	Malignant malaria
1918.						
February	563	662	744	818	—	Total admissions
	181	203	252	288	—	Benign tertian
	57	23	2	29	—	Malignant malaria
March	882	763	1059	1154	1249	Total admissions
	348	389	376	387	310	Benign tertian
	12	17	5	7	3	Malignant malaria
April	1510	1443	1509	1172	—	Total admissions
	410	337	380	392	—	Benign tertian
	0	2	0	0	—	Malignant malaria

In conclusion I wish to offer my sincere thanks to my various colleagues in the Balkans during 1916, 17 and 18 for the assistance rendered on numerous occasions. I am especially indebted to Captains C. E. C. Ferrey, O.B.E., Cecil Clarke and Arthur Wilkin, R.A.M.C. and to Corporal F. Panichelli, M.S.M., for constructing suitable apparatus from the limited material at his disposal.

#### SUMMARY.

The chief facts learnt from these investigations on blackwater fever are summarised briefly here in the order in which they are detailed in the sections.

1. True jaundice, due to the presence of bile pigment in the plasma, occurred in a high percentage of the cases which ended fatally. Haemoglobinaemia, varying from a deep red coloration of the plasma to a faint tinging, occurred in the acute stages of the disease. There was no evidence of auto-agglutination or auto-haemolysis. The fragility of the red cells was unaltered. No relationship between syphilis and blackwater fever was obtained. Spirochaetes were not demonstrated in the blood in any instance. In the hundred cases in this series, a malarial history was obtained in every instance, and without exception the infection was contracted in the Balkans during 1915-1918. The only blood parasites found were *Plasmodium vivax* and *Plasmodium falciparum*.

2. The most important tissue changes were as follows: Fatty degeneration of the myocardium. Reduction of the fat lipid content of the adrenal gland. Enlargement of the Malpighian corpuscles of the spleen due to various changes in the endothelial centres. Recent or chronic perisplenitis was present in every instance. The other changes in the spleen were numerous and variable and due to blood destruction and malaria. Haemorrhages in the liver tissue and foci of central necrosis, in which reactionary changes had occurred, were met with in many instances. Inspissation of the bile in the gall bladder and bile passages was of common occurrence.

The changes in the kidneys during the acute stages of blackwater fever were very pronounced, but complete recovery rapidly ensued during the period of convalescence without subsequent nephritis as far as the information available indicated. Spirochaetes were not found in any of the tissues in this disease. Scattered haemorrhages occurred in the most rapidly fatal cases.

3. From alcoholic and acetone extracts of the dried residue of the urine, obtained during the period of haemoglobinuria, haemolytic substances were demonstrated. From alcoholic and acetone extracts of the dried organs very active haemolytic substances were obtained, more especially from the acetone extracts.

The alcoholic extracts of the urine were more potent than the acetone in the three cases which recovered. Similar results were not obtained by ex-



tracting the dried viscera from cases other than blackwater fever, including malaria.

4. There was no evidence that quinine administered by any method during an attack of blackwater fever increased the severity or influenced the progress of the disease apart from its effects on malaria. Quinine administered to animals rendered anaemic did not excite haemoglobinaemia. Quinine was obtained from the urine by extraction, during the period of haemoglobinuria, in amounts which corresponded to those obtained in the malarial cases. Rabbits injected with quinine previous to and during the period when both ureters were ligatured failed to develop haemoglobinaemia or haemoglobinuria.

5. The urine in cases of blackwater fever rapidly returns to the normal from the period of haemoglobinuria. Clumps of red cells which possess certain characteristics were found in the urine in some cases. Clumps of red cells were of common occurrence in the tubules of the kidneys. The injection of sterilised urine from cases of blackwater fever obtained during the period of haemoglobinuria failed to produce any ill effects in animals.

6. Blackwater fever was most frequently met with during the months of March and April, at the time when the temperature was at the commencement of its upward summer rise. No evidence was obtained that blackwater fever is in any way related to anaphylaxis. It was not possible to employ any method to demonstrate whether blackwater fever is due to a filter passer.

#### REFERENCES.

- ARKWRIGHT, J. A. and LEPPER, E. K. (18. i. 1918). A series of 16 cases of Blackwater Fever occurring in the Eastern Mediterranean. *Trans. Soc. Trop. Med. and Hyg.* xi. 127.
- ARMAND-DELILLE, PAISSEAU, S. and LEMAIRE, E. (28. vi. 1917). Note sur les caractères de la bilieuse hémoglobinurique observée chez les paludéens de l'Armée d'Orient. *Bull. et Mém. Soc. Méd. des Hôpit. de Paris*, xli. 773.
- BARRATT, J. O. W. and YORKE, W. (1909). An investigation into Blackwater Fever. *Ann. Trop. Med. and Parasitol.* iii. 1.
- BIJON, R. (x. 1915). Etude expérimentale chez l'homme de l'influence de la quinine dans la pathogénie de la fièvre bilieuse hémoglobinurique. *Bull. Soc. Pathol. Exot.* viii. 597.
- BURKITT, R. W. (20. xi. 1915). Blackwater Fever. *Lancet*, ii. 1138.
- (20. iv. 1919). The Treatment of Blackwater by intravenous injections of gum arabic.... Pamphlet circulated by Consultant in Malaria. War Office.
- DUDGEON, L. S. (1906). A Study of the various changes which occur in the tissues in acute diphtheritic toxæmia. *Brain*, xxix. 227.
- (i. 1918). Certain observations on action of Bile and Bile Salts with and without the addition of the salts of calcium on animal red blood corpuscles. *Journ. of Hygiene*, xvi. 240.
- (xi. 1919). On the effects of injections of quinine into the tissues of Man and Animals. *Ibid.* xviii. 317.
- and CLARKE, C. (vii. 1919). An investigation on Fatal Cases of Pernicious Malaria caused by *Plasmodium falciparum* in Macedonia. *Quart. Journ. of Med.* xii. 372.
- FIESSINGER, N. (1918). *Compt. Rend. Soc. de Biol. Paris*, lxxxi. 38–40.

- FLETCHER, W. (1913). Report on Blackwater Fever. Federated Malay States. *13th Annual Report of the Institute for Medical Research, Kuala Lumpur*, 1913.
- HANNEMANN, K. (1918*a*). *München. med. Wochenschr.* LXV. 615-619.
- (1918*b*). *Ibid.* LXV. 665-669.
- HARTMANN, H. and ZILA, L. (1918). The Fate of Quinine in the Organism. *Arch. Exper. Pathol. and Pharmacology*, LXXXIII. 221.
- HATORI, J. (i. 1915). Blackwater Fever in Formosa. *Ann. Trop. Med. and Parasitol.* VIII. 641.
- HUNTER, W. (1901). *Pernicious Anaemia*. London: Griffin and Co.
- (1908). "Jaundice"; "Diseases of the Liver" in Allbutt and Rolleston's *System of Medicine*, IV. Part 1.
- MACLEAN, H. (1918). *Lecithin and Allied Substances. The Lipins. Monographs on Biochemistry*. Edited by Plimmer and Hopkins. London: Longmans.
- MATKO, J. (17. i.-14. ii. 1918). Ueber Wechselbeziehungen zwischen Harn und Chinin in der Hämolyse. *Wien. klin. Wochenschr.* XXXI. 65, 130, 188.
- MUIR, R. and DUNN, J. S. (1915*a*). The retention of iron in the organs in haemolytic anaemia. *Journ. of Pathol. and Bacteriol.* XIX. 417.
- (1915*b*). The absorption of iron from the organs after haemolysis. *Ibid.* XX. 41.
- and MCNEE, J. W. (1912). The anaemia produced by a haemolytic serum. *Ibid.* XVI. 410.
- NAPIER, A. H. (x. 1913). Is Syphilis a factor in Blackwater Fever? *Indian Med. Gaz.* XLVIII. 389.
- NIERENSTEIN, M. (iii. 1919). Haemoquinic acid—a new disintegration product of quinine, present in the urine especially in "Blackwater" Fever. *Journ. Roy. Army Med. Corps*, XXXII. 215.
- PAISSEAU and LEMAIRE (1916). *Bull. Acad. de Méd. de Paris*, LXXVI. 300.
- PHEAR, A. G. (1920). Some clinical notes on Blackwater Fever in Macedonia. *Journ. Roy. Army Med. Corps*, XXXIV. 1.
- PLEHN, A. (9. vii. 1914). Ein Beitrag zur Kenntnis der akuten hämolytischen Malaria. *Deutsche med. Wochenschr.* XL. 1414.
- PORAK, R. (31. v. 1918). Bileuse hémoglobinurique paludéenne et auto-anaphylaxie. *Bull. et Mém. Soc. Méd. des Hôpit. de Paris*, XLII. 559.
- DE RAADT, O. L. E. (v. 1917). Die Komplementogene Wirkung von Chinin im Zusammenhang mit dem Entstehen des Schwarzwasserfieberanfalles. *Archiv f. Schiffs- u. Tropen Hyg.* XXI. 149.
- SEYFARTH (1918). *Ibid.* XXII. 128-145.
- STANNUS, H. S. (iv. 1913). The Etiology of Blackwater Fever. *Trans. Soc. Trop. Med. and Hyg.* VI. 181.
- STEPHENS, J. W. W. (1907). See Allbutt's *System of Medicine*, II. Part 2, p. 289.
- (1913). Studies in Blackwater Fever. *Ann. Trop. Med. and Parasitol.* VII. 479.
- YORKE, W. (1911). Passage of Haemoglobinaemia through the kidneys. *Ibid.* V. 401.



## SOME FACTORS IN THERMAL SANITATION IN THE TROPICS.

BY GEORGE WALTER GRABHAM, M.A., F.G.S.

(With Plate I, 3 Charts and 3 Text-figs.)

### INTRODUCTORY.

IN torrid regions, thermal environment is of very great importance as regards comfort and certainly influences the health and efficiency of white men. The human race may have arisen under the influence of climates similar to those now prevailing in the Tropics where indigenous peoples sojourn in an almost natural state with little protection against either heat or cold. The European brings with him his native conventions as regards houses and clothing, developed, in the case of Britons, in quite northern regions, where the human race requires protection against cold. These may be more or less unsuitable for tropical heat but, being conventions, they cannot be lightly thrown aside. European clothes afford protection against disease-carrying insects, poisonous stings and bites, and the question arises as to the best way to modify them to suit the hot climate. In any case, the northern European in the Tropics is in an abnormal climatic environment and, though he may survive a long time, there can be little doubt that he rarely enjoys his natural robustness of health with the result that even trivial ailments assume serious aspects. It appears that there is a much narrower margin of health stability, and proportionately greater care is required if the system is to be kept within the limits. Mr Elsworth Huntingdon<sup>1</sup> has adduced evidence suggesting that white races, even if they are able to survive within the torrid zone, undergo deterioration.

The present paper deals with the heat absorbing properties of various materials and fabrics, and more briefly with experiments on aspects of the translucency and porosity of materials used for clothing. These subjects are introduced by an account of the experiment which led the way to the investigations.

While travelling in Kordofan in 1913, I used a khaki shirt and found, in the sun, that it felt a great deal hotter than did white ones to which I had been accustomed. No garments were worn either under or over the shirts and their respective materials were clearly the principal factors to examine. At the village of Um Semeima, on Feb. 22, the white and the khaki coloured shirts were laid out on sandy ground in the full light of the midday sun.

<sup>1</sup> Huntingdon, E. (1915), *Civilisation and Climate*, Yale.

They were folded in the ordinary way and placed with pockets upwards so that a good many folds of cloth insulated the upper layers from the ground. Only one thermometer was available. Its bulb was placed for a period of a few minutes in the pocket first of one shirt and then in the other and showed:

Under khaki	Under white
55° C.	50° C.

The thermometer was cooled in water between these observations. Next, it was left in the khaki shirt and the mercury rose to 60° C. Then, while still recording 60° C., it was transferred to the white shirt and the temperature quickly fell to 57° C. The instrument was not allowed to remain longer and was returned to the khaki shirt where the mercury rose steadily until 67° C. was recorded. It was then again taken and replaced in the white shirt where the mercury fell quickly to 60° C., later to 57° C., and still later to 55° C. during a total space of about 20 minutes. The thermometer was then returned to the khaki shirt and at 3 o'clock, after having remained half an hour, it recorded 58° C. The experiment was begun about 1 o'clock and the power of the sun's rays was decreasing rapidly at the time of the last observation. The manner in which the record fell on transference of the thermometer from the khaki shirt to the white afforded incontrovertible proof of the relative coolness of the white in regard to absorption of the sun's rays. Even under the white cloth, the temperatures attained, 50° C. to 55° C. (or 122° F.—131° F.), are quite high enough for comfort and it seems probable that those under khaki drill, in this instance rising to 67° C. (152·6° F.), are not salutary. Needless to say, the khaki shirt was promptly put aside and I have not since worn a garment of that tint in tropical sunlight. There is little wonder that wearers of khaki find thick spine pads desirable. With the results of this simple experiment before us and having regard to the extent that khaki coloured clothes are worn, it seems marvellous what the human system can stand in the way of heat. In point of fact, the heat of midday sun is most often avoided by Europeans; a good deal of travelling is done at night and outdoor duties as far as possible are relegated to hours when the sun is low.

The study of absorption of solar heat by various kinds of cloth<sup>1</sup> has been pursued on lines arising from the first experiment. The earliest step was to obtain a series of thermometers so that temperatures of the several samples could be obtained simultaneously. The first lot proved unsatisfactory by reason of the large differences in their readings at higher temperatures. Eventually a set of fifteen chemical thermometers was collected and comparisons at various temperatures showed that their errors did not exceed 0·5° C. from the mean over a range from 20° C. to boiling point. The readings of these thermometers have been adopted without correction. Conceivably better sets might have been obtained and more refined methods adopted, but the experiments have been carried out with regard to their bearing on ordinary applications and even if the error amounted to a whole degree it would appear to have no consequence in the conclusions. If the temperatures due to absorption of solar rays of two materials differ by only a single centigrade degree, other factors, cost and appearance, are likely to weigh more in selection.

<sup>1</sup> In this connection refer to Nuttall (II. 1919) *Parasitology*, XI. 205, wherein the absorption of radiant heat by cloth of various colours is discussed and the older experiments of Krieger are cited.—Ed.



## CLOTHS.

The general method pursued with both cloths and paints has been to set out the items, each with a thermometer in place, and record the readings at half-hour intervals throughout the sunnier part of the day. The hotter season of the year is the more interesting time for these experiments and calm days were selected so as to avoid undue convection effects due to wind. Calls of duty limited the number of days with the requisite time available and the free days have often been unsuitable owing to season or weather. This accounts for the length of time over which the experiments have been spread. Moreover, as the studies proceeded, improvements and extensions were made so that the latest experiments present the fullest results.

The cloths that have been dealt with are detailed in the following list:

## LIST OF CLOTHS.

I. BLACK, thin lining. Purchased locally. A smooth cotton cloth shiny on one surface and dull, but with little nap on the other surface. The dull surface was exposed.

II. BLACK, imitation serge. Purchased locally. A cotton cloth with one surface smooth, the other with nap. Used for cheaper native cloaks. The smooth surface was exposed.

III. BLACK serge. Purchased locally. A thin woollen cloth with little nap. Used for more expensive native cloaks.

IV. DARK BLUE "Zerak." Purchased locally. A dark blue Manchester cotton cloth dyed in Egypt and commonly worn by boatmen on the middle reaches of the Nile.

V. KHAKI, thick, unwashed, cotton drill. The same cloth as VI, but new. The inner surface was exposed. The washed sample was slightly lighter in shade and was rougher as it had not been ironed.

VI. KHAKI, thick, washed, cotton drill. Uniform cloth of the ranks of the Egyptian army. The test piece was a coat which had been washed not more than six times. There was no difference in tint between the two surfaces and the inner was exposed.

VII. KHAKI serge, London. A woollen cloth of medium thickness and tint very near that of the Egyptian army drill. The outer face with a distinct nap was exposed.

VIII. KHAKI "solaro," London. The well-known cloth with a red coloured inner surface. In the sample examined, there was no khaki colour among the constituents and a magnifying glass showed the appearance was due to red, pale blue and yellow strands which are interwoven. The resulting tint was rather lighter than that of VI.

IX. KHAKI Bedford cord, London. A thick woollen cloth with a ribbed surface which was exposed. The colour was a slightly warmer brown than in the preceding samples.

X. KHAKI, thin drill, London. A cotton cloth of officers' weight as compared with the men's drill of samples IV and V. The colour was slightly lighter than that of any other of the khakis. There was no difference in shade between the two sides and the inner side was exposed.

XI. PALE BLUE, "lebeni." A cotton cloth often worn by craftsmen in Egypt and the Northern Sudan. The test piece was an old and much washed garment.

XII. WHITE DUCK, London. A linen cloth of medium thickness.

XIII. WHITE DRILL, washed. A thick cotton cloth used for uniforms in the Egyptian army. The test piece had been washed several times and was not as smooth or as white as the new material XIV.

XIV. Similar to XIII but new.

The cloths have been arranged approximately in the order in which they were found to absorb solar heat. Only the same set of samples has been used so that complications due to variations in a similar pattern might be avoided. Full experiments were made on four occasions but certain selected samples have been tested at other times. The results have been consistent and it is only necessary, here, to present the details of the latest experiment, and summarise the others. Precautions have always been taken against external influence such as transmission of heat from the surroundings. A common, local, rope-strong bedstead afforded a convenient platform standing about 50 cm. above the ground. This was covered with a doubled woollen blanket to insulate the experimental cloths against heat disturbances from below. The blanket was red and in its turn was covered with a white sheet to prevent disturbance due to absorption of solar heat by the coloured material. Most of the test-pieces were lengths of cloth obtained for the purpose and folded so that they consisted of at least six layers of cloth and the smallest covered an area of 24 cm.  $\times$  18 cm. and thus was large enough to ensure that a centrally placed thermometer was unaffected by heat transmitted from the periphery. Even so the precaution of the white background was clearly desirable as it reduced solar heating which would certainly have affected the supernatant air. The thermometers were each inserted below the uppermost folds so that the bulbs were covered by single thicknesses of the cloth and separated from the support by at least five thicknesses of the same cloth. In opaque kinds of cloth, the number of folds below the bulb can hardly be important but in translucent kinds these additional layers must absorb rays that had traversed the outer layer. It is maintained that in these experiments the five interposed layers have been enough to prevent the thermometer being affected by absorption of rays which traversed the test-piece and were absorbed by the support.

Table I, p. 249, gives the results of the readings on May 31, 1918. The experiment was set out at 9 a.m. in the manner that has been described. The sky was cloudless throughout the time and there were light easterly breezes with calm intervals. The recorded maximum in the official screen for the day was 108.6° F. or 42.5° C. A thermometer with its bulb freely exposed in the air and sunshine was placed alongside the test-pieces. Its readings are included in the table. These figures have been plotted in Chart I, which displays the results graphically. The black samples gave the highest temperatures rising to 90° C. or 194° F. The khaki cloth records form a group rising to between 75° C. and 80° C. or 167° F. and 176° F., and this group is traversed by the records of the blue samples. The dark blue gave a higher temperature than any of the khakis and has given a curve of a type distinctly different from most of the other cloths. The influence of even a light shade colour is shown by the record of the pale blue "lebeni" which yielded temperatures approximately to the cooler khakis. Judging by eye the "lebeni" would certainly be chosen as a fainter shade than the khaki in its respective tint. The differences of



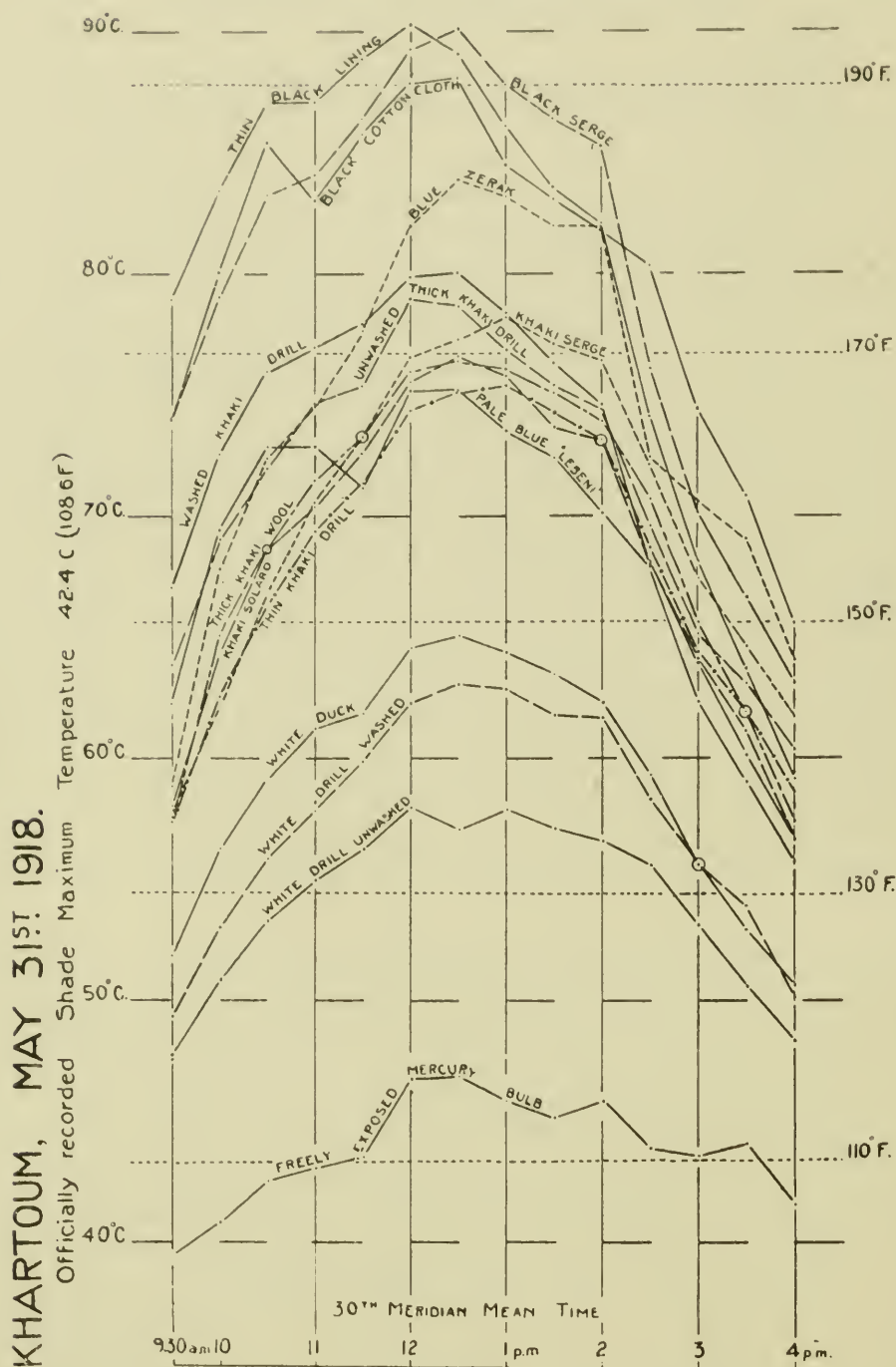
Table I. *Cloths.*

Khartoum, May 31, 1918.

Time	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	Thermometer bulb freely exposed	Conditions
9.30 a.m.	78.9	74.0	73.9	58.8	67.0	63.8	57.8	58.0	57.5	57.3	62.2	51.8	49.3	47.7	39.4	Light breeze
10.00	83.4	80.0	79.0	67.8	72.5	69.0	62.1	64.3	65.0	62.5	69.4	56.2	53.0	50.8	40.8	Light breeze
10.30	87.0	85.4	83.2	72.3	75.9	71.9	66.8	68.6	68.6	66.3	72.8	59.1	55.8	53.2	42.5	Light breeze
11.00	87.0	83.0	84.0	74.5	76.9	74.6	70.5	70.3	71.4	69.3	72.8	61.2	57.8	54.9	43.0	Light breeze
11.30	88.8	85.7	86.3	77.6	77.9	75.3	73.2	72.5	73.2	71.3	71.1	61.8	59.8	56.2	43.5	Still
12.00	90.2	87.8	89.2	81.9	79.8	78.9	76.5	75.5	75.9	74.3	75.1	64.5	62.2	57.9	46.7	Light breeze to still
12.30 p.m.	89.0	88.0	90.0	83.8	80.0	78.6	77.2	76.5	76.3	75.0	75.1	65.0	63.0	57.0	46.8	Still
1.00	86.0	84.4	87.7	83.1	78.3	76.8	78.1	75.7	76.0	75.3	73.4	64.3	62.8	57.8	45.8	Still
1.30	83.5	83.0	86.3	81.9	76.2	75.3	77.0	73.6	75.0	74.2	72.3	63.4	61.7	57.0	45.1	Still
2.00	82.0	81.7	85.2	81.9	74.5	74.3	76.3	73.0	73.8	73.0	70.0	62.2	61.6	56.5	45.8	Light breeze
2.30	74.0	80.2	76.0	72.3	68.0	69.5	72.0	67.9	70.6	68.6	67.8	59.2	58.2	55.5	43.8	Still
3.00	68.0	74.3	70.0	70.5	63.8	65.0	67.3	64.0	65.5	64.3	62.2	55.5	55.5	53.0	43.5	Light breeze
3.30	63.6	70.7	66.5	68.9	60.0	63.0	64.5	61.0	61.8	61.8	58.9	52.8	53.8	50.5	44.0	Still
4.00	59.0	65.5	63.0	63.9	56.5	60.2	61.6	56.5	57.3	58.5	55.6	50.5	50.0	48.2	41.5	Still
Mean 9.30 a.m. to 4.00 p.m.	80.0	80.3	80.0	74.2	71.9	71.1	70.0	68.4	69.1	68.0	68.5	59.1	57.5	54.0	43.7	—
Mean 11.00 a.m. to 1.00 p.m.	88.2	85.8	87.4	80.2	78.6	76.8	75.1	74.1	74.6	73.0	73.5	63.4	61.1	56.8	45.2	—

temperature among the various khakis are mainly due to differences of tint, some of the test pieces being distinctly lighter coloured than others. The white cloths gave very much lower temperatures and the coolest did not

CLOTHS.



## Chart I

reach 58° C. or 136.4° F. Fahrenheit figures are marked on the chart so that values on that scale can be estimated.

Chart I shows irregularities in some of the temperatures. For instance several of the test-pieces showed falls between 10.30 and 11.30 while the rest



continued to become hotter. A similar effect, though there is no fall, occurs in the same part of the record of the freely exposed thermometer and presumably these variations are due to atmospheric conditions. Variations of the same kind have been observed in the course of most of the experiments and they are referred to later on.

Similar remarks can be made about the other experiments so far as the number of test-pieces included allow comparisons. Attempts have been made to try and correlate the different results by reducing the figures to percentages in terms of the range between the hottest and the coolest kinds of cloths. Correlation with ideal test-pieces would allow each cloth to be given its definite place in the series and might enable results, perhaps from single observations, on different occasions to be combined. At the outset a difficulty occurs because the same black cloth is not always the hottest and results are less satisfactory if the whole range of observations from 9.30 a.m. to 4 p.m. is considered. The necessary ideal test-pieces appear to be impossible, for temperatures in sunshine are not entirely dependent on colour but are influenced by surface texture and the rate at which heat can be lost by convection. The influence of this factor is suggested later on, among the results of experiments in which the cloth was covered by glass. An unsatisfactory form of correlation might be arrived at on the assumption that certain black and white test-pieces give temperatures say  $5^{\circ}\text{C}$ . higher and lower respectively than the theoretical ideal and referring the intermediate cloths to this increased range. To illustrate by examples, taking thin black lining I and new white drill XIV as our standards the following figures can be arrived at on the basis of some of the observations made on May 31, 1918. The readings at 11.30 a.m. and 1 p.m. have been selected and have yielded the following figures:

	11.0 a.m.		Natural %	1.0 p.m.		Natural %
	Reading	Diff.		Reading	Diff.	
Black lining	87.0	32.1	100.0	86.0	28.2	100.0
Black serge	84.0	29.1	90.7	87.7	29.9	105.6
Khaki serge	70.5	15.6	48.6	78.1	20.3	70.8
White drill	54.9	00.0	00.0	57.8	00.0	00.0
	11.0 a.m. Reading	Assumed range	%	1.0 p.m. Reading	Assumed range	%
Ideal black	—	42.1	100.0	—	38.2	100.0
Black lining	87.0	37.1	88.2	86.0	33.2	86.9
Black serge	84.0	34.1	81.1	87.7	34.9	91.3
Khaki serge	70.5	20.6	49.0	78.1	25.3	66.2
White drill	54.9	5.0	11.9	57.8	5.0	13.1
Ideal white	—	00.0	00.0	—	00.0	00.0

These are enough to show that there is very little to be gained by applying percentages, either on natural or assumed bases, to single sets of observations. In practice a more general comparison is desirable and an aspect of this can be obtained simply by taking the mean of several observations. In the particular experiment presented here, these range from 9.30 a.m. to 4.0 p.m.

and the means for that set of observations on each cloth is given in the last line but one of the table. Such a range of observations includes three distinct periods. During the first, the temperatures are rising fast. During the second the temperatures alter less, absorption is greatest and the highest readings are obtained. Finally during the last period the temperatures are falling rapidly. The most simple absorption effects must occur during the middle period which can be regarded as extending from 11.0 a.m. to 1 p.m. and the lowest line of the table gives the means for observations during that time. The 11 a.m.–1 p.m. period probably forms the most satisfactory basis for comparisons and it is important if this conclusion can be established. With the adoption of black and white standard cloths, it would permit the comparison of the absorptive properties of various cloths by observations extending over periods of two or two and a half hours instead of much longer times.

A digest of three experiments with cloths is given in Table II, p. 253. For the most part the figures are limited to the means for the 11 a.m.–1 p.m. period and percentages based on these means. The period 11.30–1.30 p.m. has been taken for the 13. iv. 17 experiment. The readings were only begun at 11.30 a.m. but the highest temperatures occurred late, so the later period seems to be comparable with the 11 a.m.–1 p.m. time of the other experiments. For the experiment of 31. v. 18 the means and percentages of the 9.30 a.m.–4 p.m. period are also given. In the last column to the right are means deduced from the 11 a.m.–1 p.m. period including the 1.30 p.m. observation of 13. iv. 17, of all three experiments, and next to it are a set of percentages based on these mean temperatures. The blue cloths were not represented in all the experiments but their percentages are based on those in which they were represented, and the temperatures corresponding to these percentages are shown among the others but are distinguished by brackets. These two temperatures doubtless approximate to the means which would have been obtained had those test-pieces been exposed on all three occasions.

On two other occasions, experiments included three of the test-pieces and taking the means of the 11 a.m. to 1 p.m. observations for the percentage for the thin khaki drill, the figures are as follows:

Date	Black lining, I	Thin khaki drill, X		New white drill, XIV
		Mean	%	
7. vi. 18	86.9	73.1	53.4	29.6
14. vi. 18	85.1	73.2	53.5	25.6

The agreement of the percentages obtained on these two occasions both between themselves and with the means in the table affords a strong measure of support for this method of making comparisons. On the other hand inspection of the percentages obtained on the three occasions shows a great deal of divergence particularly at the ends of the scale among the whites and the blacks. These differences appear to be due to the surface texture and the atmospheric conditions prevailing during the experiments. On a very still



Table II. *Digest of Temperatures of Cloths.*

Cloths	14. v. 15			13. iv. 17			31. v. 18			Mean of three experiments		
	11 a.m.-1 p.m.			11.30 a.m.-1.30 p.m.			9.30 a.m.-4 p.m.			11 a.m.-1 p.m.		
	T.	%		T.	%		T.	%		%	T.	
I Black, thin lining ...	91.6	100.0	...	75.2	100.0	...	80.0	100.0	...	100.0	85.0	...
II Black imitation serge ...	89.7	93.2	...	76.3	104.7	...	80.3	101.1	...	96.0	83.9	...
III Black serge ...	85.9	79.5	...	75.5	101.3	...	80.0	100.0	...	92.7	83.0	...
IV Dark blue "zerak" ...	—	—	...	71.7	84.8	...	74.2	77.7	...	78.8	(79.2)	...
V Khaki, thick drill, washed ...	81.0	61.9	...	68.2	69.7	...	71.9	68.8	...	67.1	76.0	...
VI Khaki, thick drill, new ...	83.2	69.8	...	67.0	64.5	...	71.1	65.8	...	66.1	75.7	...
VII Khaki serge ...	82.5	67.3	...	67.8	68.0	...	70.0	61.5	...	63.9	75.1	...
VIII Khaki "solaro" ...	80.1	58.6	...	64.5	53.7	...	68.4	55.4	...	55.8	72.9	...
IX Khaki Bedford cord ...	79.0	54.7	...	64.7	54.5	...	69.1	58.1	...	55.5	72.8	...
X Khaki, thin drill ...	77.2	48.2	...	66.7	63.2	...	68.0	53.8	...	53.6	72.3	...
XI Pale blue "lebeni" ...	—	—	...	—	—	...	68.5	55.8	...	53.2	(72.2)	...
XII White duck ...	67.0	11.5	...	55.5	14.7	...	59.1	15.7	...	15.7	61.9	...
XIII White drill, washed ...	64.0	00.7	...	53.4	5.6	...	57.5	13.5	...	6.9	59.5	...
XIV White drill, new ...	63.8	00.0	...	52.1	0.0	...	54.0	00.0	...	0.0	57.6	...
Thermometer with bulb freely exposed ...	46.7	—	...	—	—	...	43.7	—	...	—	—	...
Officially recorded maximum	44.6	—	...	38.5	—	...	—	—	...	—	—	...

day, losses by convection must be smaller. While under windy conditions, a cloth with a nap would hold the air and not lose heat as rapidly as a smooth or well-ironed cloth. Even though the method of percentages is by no means perfect it appears to offer a useful means of comparison. The figures certainly indicate the relative amounts of heat that persons wearing the different cloths would have been subject to in the sunshine on those particular days.

Even if it were possible to test a very large number of cloths at the same time, the result would be influenced by the atmospheric conditions and would certainly be of less value than a series based on means of experiments on different occasions from 11 a.m. to 1 p.m. on smaller sets of test-pieces. Doubtless, the more the observations can be multiplied, the greater the value of the percentages based on them.

#### PAINTS.

Much that has been said about the testing of cloths applies to paints and colour washes but these offer simpler cases of solar heating. The surfaces, including shiny and dull kinds, are practically uniform compared with the differences of texture that exist among cloths.

For the purposes of experiments, cylindrical tin flasks 12½ cm. long and 7½ cm. in diameter were adopted. They were provided with necks to accommodate corks through which thermometers were inserted so that the bulbs

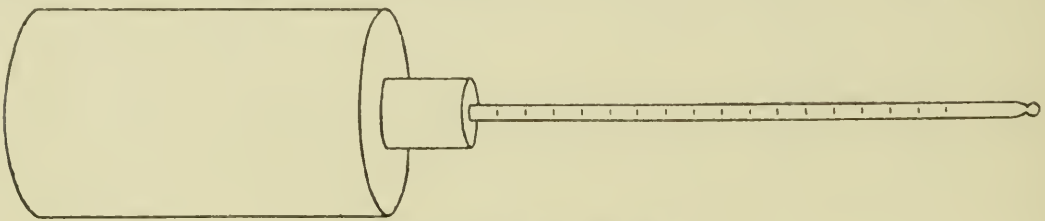


Fig. 1. Paint testing flask.

were placed freely at about the centres of the flasks. The flasks were laid on their sides and in order to give them stability a four-ounce volume of clean sand was measured into each. The amount of sand was not enough to come in contact with the thermometer bulb and, as the quantities were similar, it can hardly have influenced comparison of the temperature effects due to the paints.

The principal experiments were conducted at Halfa in 1916. The flasks were laid out on a white sheet resting on a doubled woollen blanket to eliminate, as far as possible, disturbing factors due to heat absorption by the surroundings. They were set out with intervals of about 30 cm. between them and arranged in order of their apparent tints so that the lighter coloured flasks were next each other and distant from the darker ones. These precautions were taken to reduce effects due to radiation from one flask to another such as might have interfered had a black flask been near a light coloured one. Both the Sudan Railway Dept. and Public Works Dept. kindly undertook the painting of sets of flasks with colours in common use. Besides



the immediate interest of testing such colours, there was the advantage of having them applied by the very workmen and in the same way as in ordinary practice.

Standards of reference were provided by other flasks, the white being coated with a lime wash which gave a dead white surface while the other was coated with a mixture of lamp-black and varnish which dried with a dull black surface. These standards have always been included and the results of experiments with two sets of paints are presented here.

Public Works Departments, PAINTS, Halfa, 15. v. 16. Maximum temperature in official screen, 44° C.

Time	Black standard	Cement wash	Bright red	Dull red	White enamel	White standard
10.00 a.m.	64.4	58.2	56.0	56.0	49.6	47.0
10.30	70.2	63.7	61.0	61.4	54.2	51.8
11.00	74.0	67.5	64.6	65.2	58.2	55.0
11.30	78.0	73.0	69.4	70.3	60.8	57.1
Noon	72.8	68.0	65.2	65.7	57.5	54.5
12.30 p.m.	77.7	73.0	69.6	70.0	60.2	56.9
1.00	74.6	70.7	67.5	67.5	58.8	55.5
4.10	61.5	56.8	56.0	54.0	47.5	44.2
Mean of experiments on 14 and 15. v. 16	69.5	65.3	(62.2)	61.9	54.7	52.0

Sudan Government Railways, PAINTS, Halfa, 2. vi. 16. Maximum temperature in official screen, 40° C.

Time	Black standard	Black paint	Brown paint	Green paint	Grey paint	Khaki paint
		(9)	(8)	(4)	(7)	(6)
Noon	61.0	60.7	60.0	59.5	58.0	56.5
12.30 p.m.	66.2	66.8	63.2	63.8	61.0	59.1
1.00	63.1	63.1	62.2	61.2	59.4	58.0
1.30	63.2	63.1	61.7	61.5	59.3	58.3
2.00	63.2	62.9	61.0	60.0	58.4	57.0
2.30	62.0	61.4	59.7	59.0	57.4	56.0
3.00	63.2	63.0	61.3	60.3	59.0	57.5
3.30	61.0	60.2	59.4	58.0	56.8	55.9
Mean	62.9	62.5	61.4	60.4	58.7	57.3
Time	Scarlet paint	Straw paint	Cream paint	Cream enamel	White enamel	White standard
	(10)	(5)	(2)	(1)	(3)	
Noon	53.5	51.3	48.8	48.9	—	43.5
12.30 p.m.	56.3	54.2	53.0	53.0	50.2	46.6
1.00	55.2	53.0	51.3	51.3	49.3	45.8
1.30	55.2	53.8	52.0	51.8	50.0	46.2
2.00	54.2	52.8	51.0	50.7	49.2	45.5
2.30	53.5	52.0	50.5	50.6	49.0	45.5
3.00	54.6	53.2	51.5	51.0	49.5	46.0
3.30	53.0	51.6	49.9	49.4	47.8	43.0
Mean	54.4	52.7	51.0	50.8	49.1	45.3

These results are expressed in Chart II.

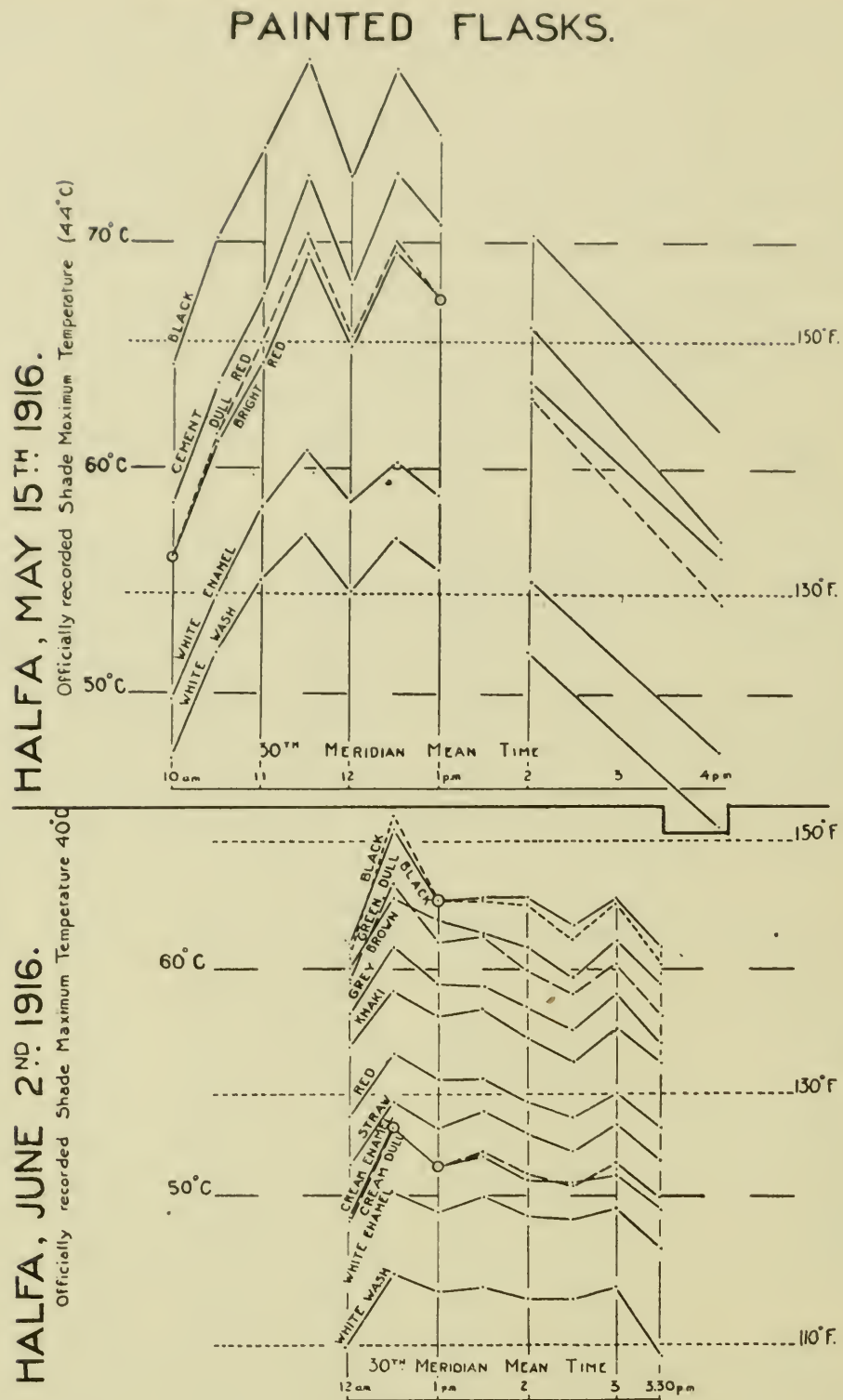


Chart II.

The application of the method of percentages has been discussed in connection with comparisons of temperatures given by cloths. The painted flasks have proved far more amenable to the method as the following figures show.

The only points about these figures that require explanation are the two means. The arithmetic mean is simply that of the column of figures above it while the lower figure is based on the range of the mean temperatures given



*Percentages.*

	Black	Brown	Green	Grey	Khaki
Time	(9)	(8)	(4)	(7)	(6)
Noon	98.3	94.3	91.5	82.8	74.3
12.30 p.m.	100.3	84.7	87.7	73.5	63.8
1.00	100.0	94.7	89.0	78.6	70.5
1.30	99.4	91.3	90.1	77.4	71.5
2.00	98.3	87.6	81.8	72.8	65.0
2.30	96.4	86.0	81.8	72.1	63.6
3.00	98.7	88.9	83.1	75.5	66.9
3.30	95.5	91.1	83.3	76.6	71.6
Arithmetic mean	98.4	89.8	86.0	76.1	68.4
Percentage of mean temperature	97.7	91.5	85.7	76.1	68.2

	Scarlet	Straw	Cream dull	Cream enamel	White enamel
Time	(10)	(5)	(2)	(1)	(3)
Noon	57.1	44.6	30.3	30.8	—
12.30 p.m.	49.5	38.8	32.7	32.7	18.4
1.00	54.4	41.7	31.8	31.8	20.0
1.30	53.5	45.4	34.9	33.7	23.3
2.00	49.2	41.3	31.4	29.4	20.9
2.30	48.5	39.4	30.3	30.9	21.2
3.00	50.0	41.9	32.0	29.1	20.3
3.30	55.6	47.8	38.4	35.6	26.7
Arithmetic mean	52.2	42.6	33.2	28.0	21.5
Percentage of mean temperature	51.6	42.0	32.4	31.2	20.4

at the foot of the previous table (p. 255) which shows the temperatures of the railway paints. The means derived in these ways do not differ materially and the percentage method of comparison is clearly very useful. It enables the results of different experiments to be correlated and the following table of them is presented:

	Number of observa- tions	%	12.30 p.m. temperatures		Excess above whitewash
Black standard	—	100.0	66.2° C.	151.2° F.	36.3° F.
Black paint (9)	8	97.7	66.8	152.2	35.2
Brown paint (8)	8	91.5	63.2	145.8	29.9
Green paint (4)	8	85.7	63.8	146.8	30.9
Grey paint (7)	8	76.1	61.0	141.8	25.9
Cement wash (P.W.D.)	14	76.0	(61.5)	(142.7)	(26.8)
Khaki paint (6)	8	68.2	59.1	138.4	22.5
Red paint (P.W.D.)	13	59.2	(58.2)	(136.8)	(20.9)
Aluminium paint	22	58.6	(58.1)	(136.6)	(20.7)
Dull red paint (P.W.D.)	14	56.5	(57.7)	(135.9)	(20.0)
Plain tin	22	54.2	(57.2)	(135.0)	(19.1)
Scarlet paint (10)	8	51.6	56.3	133.3	17.4
Straw paint (5)	8	42.0	54.2	129.6	13.7
Cream paint (2)	8	32.4	53.0	127.4	11.5
Cream enamel (1)	8	31.2	53.0	127.4	11.5
White enamel (3)	7	20.4	50.2	122.4	6.5
White enamel (P.W.D.)	14	15.4	(49.6)	(121.3)	(5.4)
White standard	—	00.0	46.6	115.9	—

The first column shows the number of observations on which the percentages in the second column are based. The figures without brackets in the third column are the temperatures actually observed in the flasks on June 2, 1916, at Halfa when the officially recorded maximum shade temperature was 40° C. It is to be noticed that the figures at that particular moment differed slightly from the order of the percentages which, as has been shown above, are based on several observations. The figures given in brackets were not observed but are interpolated on the basis of percentage values from observations at other times. The percentages appear to be consistent enough to enable the interpolated figures to be regarded with some confidence. Had the flask coated with aluminium paint been available, for instance, at Halfa when the other observations were made, there can be little doubt that a thermometer inside it at 12.30 p.m. would have recorded approximately 58° C. The fourth column gives the Fahrenheit equivalents and the last shows the amounts of the excesses in Fahrenheit degrees above the temperature of the whitewash. Thus khaki paint is seen to have been 22.5° F. hotter than the whitewash.

I regret that I am unable to give technical descriptions of the paints but it is to be noted that the green, which proved a remarkably hot colour, was the ordinary rather dark green such as is often seen on shutters and on garden furniture. The brown was of a dark tint which led one to expect high temperatures, but those given by the scarlet (10) appeared low for such a full, bright colour as the signal red of the railway. The grey was an ordinary medium shade not far removed in appearance from the cement wash which gave closely similar results. The Public Works Department red and white paints were pigmented with iron oxide and zinc oxide and the respective flasks were each treated with three coats. The dull red was the result of mixing zinc white with the red so as to match the faded appearance assumed by the red paint after prolonged exposure to sunlight. The list shows the comparative coolness of white and how even a slight tinge, such as gives a cream colour, has a marked effect on the absorption of solar heat. A polished metal surface is represented by the plain tin and is seen to give temperatures approximating to the red paints.

By the kindness of the late Mr E. W. Buckley, of the Irrigation Service, a practical test was made by whitewashing the half of the galvanized corrugated iron roof of a barge, the other half remaining in the usual condition and presenting the ordinary rather dull appearance of the weathered metal. In the sunshine of the middle hours of the day the difference between the temperatures of the two halves was very striking. The plain metal became so hot that the hand could hardly bear it while the whitewashed part remained cool and could be handled with comfort. The roof was low and in moving about under it, the radiant heat from the plain half was immediately felt as oppressive and showed the need of a helmet. Beneath the whitewashed part, on the other hand, it was possible to remain bareheaded with comfort. An



attempt on one occasion to measure the temperatures gave  $45\frac{1}{2}^{\circ}\text{C.}$  and  $33\frac{1}{2}^{\circ}\text{C.}$  respectively for the plain and whitewashed parts, but little importance is to be attached to these figures as that relating to the plain part is almost certainly too low. They indicate, however, the magnitude of the difference. Owing to the oppressive temperatures, the iron roofs of a number of similar barges have been lined with wood, but as efficient a result might have been attained by means of a coat of white paint or even whitewash so long as the latter withstood the rains.

The ordinary surveyors' ranging rod, with its sections of different colours, often provides a handy demonstration of differences in temperature in sunshine. The black part proves hot, the red rather cooler, and the white sections coolest of all.

#### BRICKS.

In February, 1915, some building bricks were tested on the same lines as adopted with the cloths and paints.

Both sun-dried and burnt bricks are in use in the Nile Valley and the coolness of mud houses as compared with those of burnt brick is common experience. For the purpose of the experiments a hole was drilled from the end so that when a thermometer was inserted it was placed about the centre of the brick. The thermometers were packed round with fine soil to keep them in place and preserve the records from disturbances due to convection. A pair of burnt bricks and a pair of sun-dried ones were used and one of each pair was left plain while the other was whitewashed. They were laid out on a board at 9 a.m. on Feb. 9, 1915, and not disturbed until after the readings on the next day. The following are the readings at 2 p.m. on the two days:

		9. ii. 15	10. ii. 15
Plain burnt brick	...	57.8	53.3
Plain sun-dried brick	...	54.3	50.4
Mud brick, whitewashed		49.5	46.9
Burnt brick, whitewashed		48.3	46.3
Official maximum	...	33.4	31.1

Possibly the hotness of the burnt brick as compared with the sun-dried structure is not due to the greater absorption alone but also to its texture. The firing must result in the grains being in more intimate connection than is the case with the sun-dried one with the result that the burnt brick takes up more heat during the hotter parts of the day and radiates it more freely in the evening. The same kind of effect is experienced in the open country. Where the desert surface is formed of loose sand, the surface cools quickly and the nights are comparatively cool even in the hottest seasons. Where, on the other hand, crystalline rock predominates, the solid stone absorbs a large amount of heat and appears to be able to radiate it during most of the night so that these are sultry and oppressive. No figures have been obtained for these conditions but if the hand is thrust into an exposed sand-dune on the afternoon of a sunny day, the heated surface layer will be found to be only about 10 or 15 cm. thick and quite cool sand will be found just underneath.

## COMBINED EXPERIMENTS.

Experiments with sets of cloths and sets of paints have been described and it now remains to show how their temperatures compare when both are exposed to sunlight at the same time. Other obvious problems arose such as the influence of the circulation of the air and a set of tests was arranged with a view to obtaining evidence on the different points. More than one trial was made before the set was adopted for the experiment now described. All the items were laid out on a white sheet resting on a doubled blanket supported on a rope-string angareeb about 50 cm. high and, as in previous experiments, the pieces giving the higher temperatures were placed towards one end of the angareeb while the cooler ones were towards the other. The set of observations, given in Table III, p. 261, was made on June 14, 1918, when the officially recorded maximum shade temperature was  $43.4^{\circ}\text{C}$ . During the earlier part of the time there were breezes but the air became still towards the middle of the day. The figures have been plotted out and Chart III presents the curves.

Three cloths were included and a comparison of the temperatures given by these with those yielded by the black standard and white standard painted flasks shows that the cloths reached their highest temperatures about 12.30 p.m. while the highest temperatures of the flasks were reached an hour later. After reaching the highest temperatures the cloths cooled down fairly rapidly while the painted flasks maintained high temperatures and only began to cool rapidly at about 3 p.m. The contrast is exhibited in the forms of the curves in the diagram. The black cloth reached a temperature nearly  $17^{\circ}\text{C}$ . hotter than the standard black painted flask and even the khaki cloth was a good deal hotter than any of the freely exposed flasks.

To obtain some evidence on the heat losses caused by free circulation of the air, a sheet of glass was laid in contact, over part of the test-piece of black cloth. Black and white painted tin flasks were enclosed in glass fronted wooden boxes made with holes at one side through which the necks of the flasks fitted fairly closely and allowed the thermometers to protrude. To minimise temperature disturbance due to absorption by the wood, it was whitewashed both on the inner and outer surfaces. During the earliest and latest hours the boxes were tilted so that the flask inside might be fully exposed to the sunlight. A considerable interval had elapsed since the original standard black and white flasks were painted; consequently control flasks were prepared and painted at the same time and with the same paints as those inclosed in the glass-fronted boxes. Comparison of the records of the control flasks with those of the standards shows that the white ones were in capital agreement, but the black control was between  $2^{\circ}\text{C}$ . and  $3^{\circ}\text{C}$ . hotter than the other at the highest temperatures. The standard black was distinctly paler, doubtless through the adherence of dust particles. Inspection of the records and curves shows that the glass-covered cloth reached a temperature



Table III. *Combined Experiments.*

June 14, 1918. Time:	9.30	10.0	10.30	11.0	11.30	12.0	12.30	1.0	1.30	2.0	2.30	3.0	3.30	4.0
Black painted, standard flask	...	56.0	60.0	61.7	64.2	65.5	64.3	66.9	66.1	67.8	68.6	68.0	63.8	59.9
Whitewashed, standard flask	...	44.8	47.0	48.2	49.5	50.9	50.8	52.2	52.5	52.3	53.2	52.0	49.6	(46.1)
White drill, new, XIV	...	52.2	55.0	56.6	58.9	60.0	59.4	60.5	58.9	57.8	56.6	53.9	50.0	(44.9)
Khaki drill, thin, X	...	60.8	67.2	70.3	71.7	73.1	74.3	74.3	72.5	71.1	69.4	64.4	57.1	51.5
Black lining, thin, I	...	74.3	80.3	82.5	86.4	84.6	85.5	85.0	84.0	81.9	79.9	75.1	64.8	55.2
Black lining, I, under glass	...	85.2	92.0	95.9	99.0	99.6	99.5	99.6	97.0	95.8	92.8	84.4	81.8	73.4
Black painted, control flask	...	58.0	62.7	64.2	67.0	67.8	66.5	69.2	67.5	70.9	71.3	69.5	70.0	61.3
Whitewashed, control flask	...	44.2	46.8	48.2	49.5	50.8	51.0	52.2	52.5	52.0	52.7	51.2	49.5	47.0
Black painted flask, boxed	...	67.0	76.5	81.2	84.2	86.2	86.6	88.0	87.4	85.5	83.7	79.7	70.1	(63.2)
Whitewashed flask, boxed	...	53.7	60.4	64.0	66.7	68.7	69.3	70.5	70.4	69.3	68.8	66.0	64.3	(56.0)
Half black and half plain tin flask	...	53.0	59.5	61.5	63.8	64.8	63.8	66.0	64.9	66.6	66.4	65.0	62.4	56.2
Half black and half whitewashed flask	51.5	57.5	59.8	62.0	63.0	62.3	62.3	64.1	63.3	64.5	64.3	62.5	62.4	54.4
Plain tin flask	...	49.8	55.0	56.8	58.7	59.1	59.0	61.0	60.5	61.0	59.5	60.0	57.6	51.8
Aluminium painted flask	...	51.0	56.0	58.0	59.5	60.0	59.9	61.5	61.0	61.2	60.8	59.2	56.1	52.2
Freely exposed thermometer	...	39.5	41.4	41.5	43.9	44.0	44.1	45.2	44.9	46.0	46.9	45.7	44.0	41.8

The test-pieces were all exposed at 9.5 a.m. At the time of the last observation some of the test-pieces were in shadow and the readings relating to these are given in brackets. The officially recorded maximum shade temperature was 43.4° C. or 110.2° F.

about 13° C. higher than the freely exposed part of the same cloth. Similar differences occurred between the boxed and freely exposed painted flasks and it is to be remarked that the glass-covered cloth proved more than 11° C. hotter than the enclosed black flask. An interesting point is that the records of the boxed flasks differ in character from those of the freely exposed ones.

The highest temperatures were attained soon after midday; they fell soon afterwards and did not maintain a rather uniformly high temperature until later, in the same way as the freely exposed ones. The result is that the

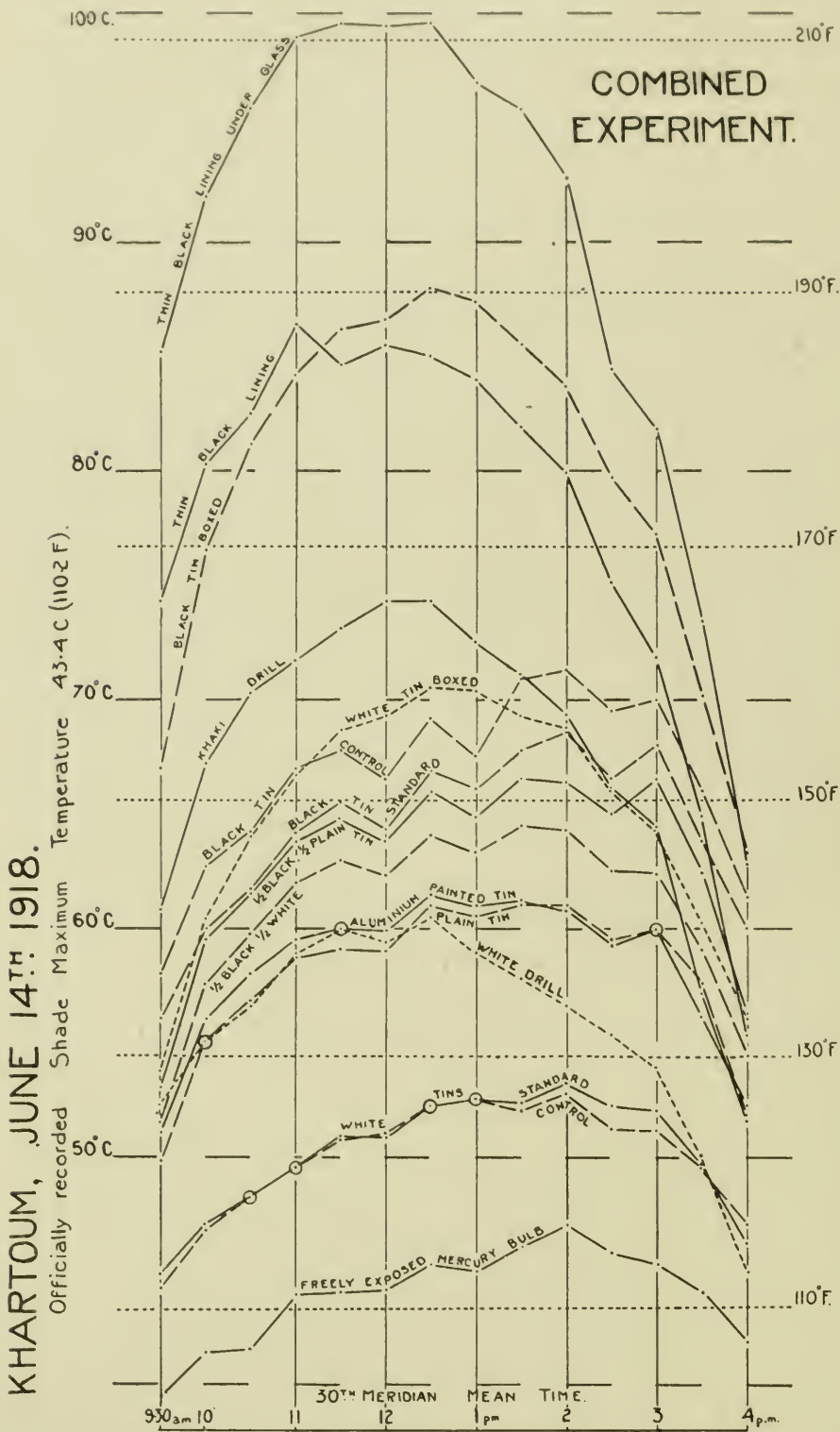


Chart III.

plotted curves for the enclosed flasks resemble in form those of the cloths and not of the other flasks. The influence of the glass is probably not as simple as appears. Apart from shielding the test-pieces from convection by the air,



it doubtless hindered the loss of heat by radiation, being opaque to the longer waves, such as the heated flasks would emit. Except for a peak in the record of the freely exposed black cloth at 11 a.m., it and that of the glass-covered part agree fairly well in form and this is supported by records from another occasion when the same test-piece was exposed. It seems as if the enclosure has achieved, in the case of the paints, a similar prevention of loss by convection as occurs in many cloths owing to the texture. The opacity of the glass to long rays has not masked the effect but merely increased it. The experiment included an ordinary thermometer with its bulb exposed freely in air and sunshine and several inches away from any other solid objects. The highest temperature was only  $3.5^{\circ}\text{C}$ . hotter than the officially recorded maximum shade temperature and, with its little mass of mercury enclosed in glass, it proved considerably cooler than any of the test-pieces. By reason of the small size of the bulb, convection was a correspondingly important controlling factor, and its temperature was mainly influenced by that of the air. It is of interest to note, therefore, the similarity between the thermometer record and those of the exposed flasks in contrast with those of the cloths and enclosed flasks from which a smaller proportion of the loss is due to convection. The experiment does not enable it to be stated what the relative proportions may be but it suggests that, at any rate, a cloth with a good nap is well protected from convection losses which must be greater in smooth and well-ironed fabrics.

The glass-covered part of the cloth attained the remarkably high temperature of  $99.6^{\circ}\text{C}$ . which appears to exceed anything recorded by black bulb radiation thermometers in this region. Records of such a thermometer have been kept for some years at Aswan and, though that is a long way from Khartoum, it is of interest to note that on two occasions in July, 1917, temperatures of  $78^{\circ}\text{C}$ . were recorded. I am able to quote these facts by the courtesy of Mr D. Watt, who thought there had been slightly higher temperatures but that  $80^{\circ}\text{C}$ . had never been reached. The unsatisfactory character of the temperatures given by black bulb radiation thermometers has long been recognised and little importance is now attached to them.

Only one-half of a flask can be in sunlight and two were prepared with a view to obtaining evidence on the influence of the side in shadow on the temperature. One was half black and half plain tin, and the other was half black and half white. During the experiment the flasks were turned round to keep the black sides towards the sun. The flask with the white on the shadow side gave temperatures nearly  $2^{\circ}\text{C}$ . lower than that with the plain tin on the shadow side which was only  $1^{\circ}\text{C}$ . cooler than the black standard.

On this occasion, the set of test-pieces was completed by the addition of a plain tin flask to represent a polished metal surface and an aluminium painted flask. The records of these two flasks have been dealt with in the section on paints. They both gave rather similar temperatures which proved to be about  $8^{\circ}\text{C}$ . hotter than those of the whitewashed flasks.

## POROSITY OF CLOTHS.

In a hot and dry climate, texture is obviously a very important factor connected with the comfort with which a cloth may be worn. Free evaporation from the skin is interfered with by the barrier formed by the cloth, and experiments were devised with a view to obtaining evidence on the hindrance caused to the passage of air and the influence this transmission factor might have on evaporation.

The apparatus used for testing transmission is diagrammatically shown in Fig. 2.

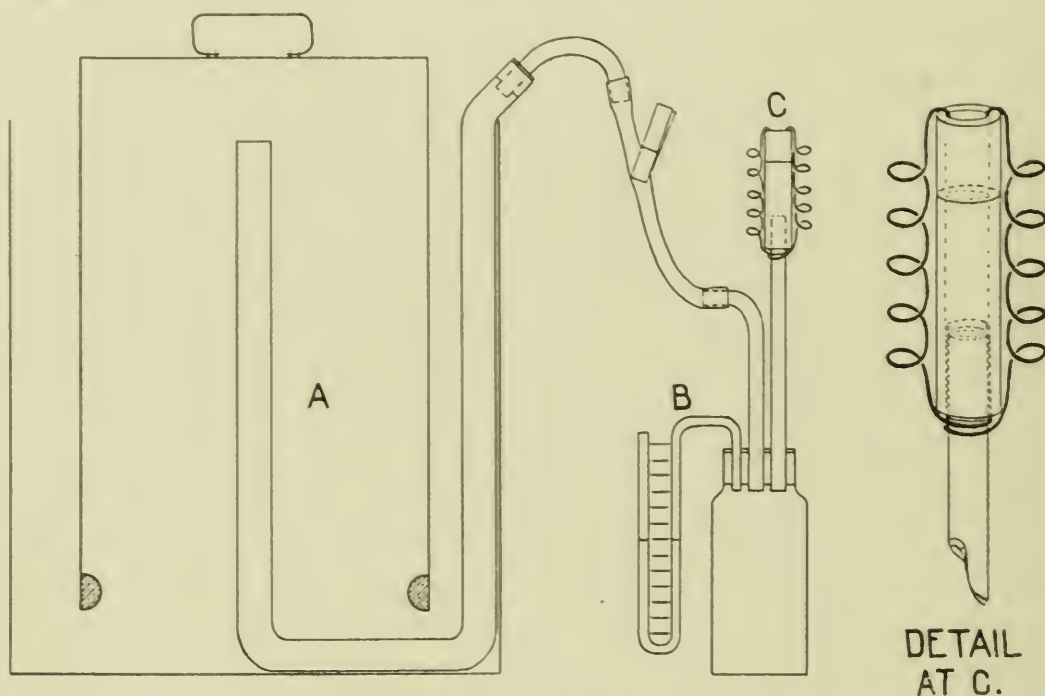


Fig. 2. Porosity apparatus.

The bottle on the right served the purpose of a three way joint to connect the aspirator (A) both with the pressure gauge (B) and the cloth holder (C). The cloth holder, shown on a larger scale, was formed of two pieces of thick glass tube 17 mm. in diameter and 12 mm. bore. The surfaces in contact with the cloth were ground flat. A strip of the cloth to be tested was inserted between the two pieces of tube. The tension of the springs served to hold it in place and probably sufficed to prevent any considerable amount of air escaping around the junction. The aspirator was provided with a scale and, for the purpose of the experiments, the time taken to sink through the distance between two marks was observed. The same marks were always used and corresponded to a volume of about 3830 c.c. The air pressure was observed on the water gauge (B). The weight on the aspirator was constant but unfortunately the passages between it and the cloth holder were not free enough to allow the same pressure to be maintained with all the different kinds of cloth tested. The pressure was a good deal less with the more porous cloths. The test-piece was moved between each observation, and in some cases other pieces of the same cloth were used.

Among the cloths selected the most notable was a piece of calico which had been a great deal worn. And, for purposes of comparison, a new piece of a similar cloth, washed only once, was included. The sample of old khaki drill was also a good deal worn as the tests indicate.



It is enough to present two sets of results in detail:

Old calico, 11. vi. 18.

Time	Pressure	Transmission factor
18	50	900
18	48	862
18½	49	905
18	42	855
20	44	880
20	44	880
18	42	755
19	43	815

Khaki drill, new, 11. vi. 18.

Time	Pressure	Transmission factor
124	63½	7860
128	63	8050
133	64	8500

The times and pressures have been used as factors and the results in the third column can be regarded as inversely proportional to the porosity.

The mean results with different cloths are as follows

	Experi- ments	Time	Pressure	Transmission factor
Calico, old, 1 ... ..	8	18.7	45.25	844.0
Calico, new, 2 ... ..	4	36.0	59.50	2142.5
Khaki "solaro," VIII ...	3	71.0	61.50	4373.0
White drill, XIV ... ..	4	86.7	60.00	5199.0
Khaki drill, thick, old, 3	3	88.7	59.30	5263.0
Khaki drill, new, V ...	3	128.0	63.50	8137.0

These figures, obtained with crude apparatus, may leave a good deal to be desired, but they serve to show the kind of differences of porosity that exist between various cloths. If the matter proves of sufficient importance it would be simple to devise means by which fabrics could be tested under definite conditions so that observations might be comparable. The present set serves to show that the old calico is more than twice as porous as the new, which again is at least double as porous as the khaki solaro and the white drill. The new khaki drill is evidently a very dense cloth.

In the tropics where comparatively light clothing is worn the human body may be regarded as encased in loose tubes of fabric, and comfort must depend largely on the rate at which evaporation can take place from the skin. Having obtained some evidence on the influence of texture on the passage of air it became necessary to see how far this might influence evaporation from moist surfaces enclosed in the cloths. This was done in apparatus referred to as lanterns, one of which is shown in Fig. 3. Each consisted of a wooden frame formed of stout discs held in position by two laths. The ends of a piece of cloth 53 × 20 cm. were stitched together so that it formed a tube that fitted fairly closely and could be drawn on to the frame. It was fastened by being tied with tapes against the discs. The cloth surface freely exposed between the discs was approximately 51 × 15.5 cm. in each case. One of the discs

was provided with a couple of eyes by which the lantern could be hung and a hole through which the tube of a piché evaporimeter passed.

The evaporimeters, kindly lent by the Physical Department of Egypt, were the standard model used in meteorological stations in Egypt and the Sudan. Allowing for the diameter of the tube and support, the paper circle

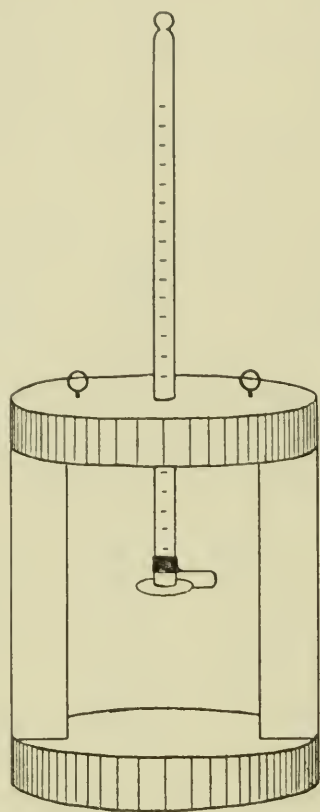


Fig. 3. Evaporation Lantern.

of the evaporimeter presented a wetted surface 12·7 sq. cm. in area. The lanterns duly fitted up were hung about 80 cm. apart on a pole in an airy verandah. *A*, on the east, may not have been quite so freely exposed as *D*, on the west. *B* was left without any cloth covering, to serve as a control on the other three. The fall of the water in the evaporimeter tubes was observed at intervals and the readings were as follows:

		10. vi. 18				11. vi. 18
		10 a.m.	2.10 p.m.	6 p.m.	10 p.m.	9 a.m.
Calico, new, washed once	<i>A</i>	1·40	2·70	3·91	5·03	7·00
Evaporimeter without cloth	<i>B</i>	1·47	4·33	6·24	8·21	13·25
Calico, old and worn	<i>C</i>	1·45	2·79	4·03	5·20	7·20
Khaki drill, new, V	<i>D</i>	1·43	2·78	4·05	5·17	7·18
Thermometer Dry bulb		35·9	38·4	36·7	35·3	33·9
Thermometer Wet bulb		21·4	22·2	23·3	21·4	23·3

The wet and dry bulb thermometers were situated in a freely ventilated room, opening from the verandah, and the readings of these are added. This experiment continued until the following day, and during the night and early morning of June 11 there was a good deal of wind. A dust storm arose



subsequently, and on opening up the lanterns it was found that dust had penetrated both the calicos, more through the old one, while almost none had penetrated the thick khaki drill. The total amounts evaporated on this and on another occasion were as follows:

		June 10-11	June 12-13	Transmission factor
Calico, new	<i>A</i>	5.60	6.43	2142.5
Evaporimeter without cloth	<i>B</i>	11.78	11.39	—
Calico, old and worn	<i>C</i>	5.75	6.48	844.0
Khaki drill, new	<i>D</i>	5.75	6.81	8137.0

The transmission factors obtained in other experiments are quoted alongside for comparison. It is remarkable that in spite of wide differences in textures, the evaporation occurs at almost similar rates through these very different types of cloth. The explanation appears to be that the water vapour passes out by diffusion and the texture of the fabric hardly makes a difference so long as it stops actual circulation but permits diffusion. Circulation of air is much hindered even by such open textures as those of the wire used for mosquito proof structures as is well known. In an exposed situation, a strong wind is very much reduced and many of us know how the few breaths of air on a calm night appear to be completely cut off by such gauze.

Doubtless there is a limit to the amount of moisture that can diffuse through a unit area of cloth and this was not reached in the experiments just described, but if the evaporimeter had been larger the rates of loss might have borne some relation to the porosity. It cannot be denied that single thicknesses of light textured fabrics form the coolest kinds of clothes, but perhaps the comfort is partly due to the clothing being loose and promoting circulation of the air by a kind of bellows action. The relation of texture to comfort is clearly a direction in which more experiments are needed.

#### TRANSLUCENCY OF CLOTHS.

Writers on tropical hygiene tell a great deal about the baneful effects of the ultra-violet solar rays and this has been expounded at length by Woodruff<sup>1</sup>. With a view to obtaining some direct evidence on the transparency of different kinds of clothing to actinic rays some sheets of ordinary photographic P.O.P. were exposed for certain lengths of time in full sunlight, behind strips of different kinds of cloths. Three sets of results have been photographed and are shown in Plate I. The cloths were as follows:

White calico	...	...	...	...	2
White calico, doubled	...	...	...	...	2a
White drill	...	...	...	...	XIV
Pale blue "lebeni"	...	...	...	...	XI
Dark blue "zerak"	...	...	...	...	IV
Black serge	...	...	...	...	III
Khaki, thin drill	...	...	...	...	X
Khaki "solaro"	...	...	...	...	VIII
Khaki serge	...	...	...	...	VII
Khaki, thick drill as VI, but much more worn	...	...	...	...	3

<sup>1</sup> Woodruff (1915), *The Effects of Tropical Light on White Men*, London.

The uppermost set shown in the figure was exposed for five minutes from 10.52 to 10.57 a.m. on June 14, 1918. The second set was exposed for 30 minutes from 12.42 to 1.12 p.m. on June 14, 1918. And the third set for 60 minutes from 11.7 a.m. to 12.7 p.m. on October 4, 1918.

The same strip of pale blue "lebeni," XI, was used in both the first and second sets and strips of thin and thick khaki drill, X and 3, are common to the second and third sets. The prints suggest that, at any rate, in the more opaque cloths, the translucency is related to the porosity. The samples of black cloth and thin khaki have both proved slightly translucent compared with the thick khaki drill which, even after being subject to considerable wear, still remained very opaque, since the photographic paper shaded by it was practically unaffected after a whole hour of exposure in clear, midday sunshine.

The pale blue cloth clearly affords good shade and the paper below it was hardly affected in five minutes though there was a marked effect after half an hour as shown by the second set. The only cloth that proved at all translucent was the white calico included in the first set, exposed for five minutes only, and even this shaded the paper considerably as is shown by comparison with the tint assumed by the unshaded paper. For the rest the cloths evidently cut off a very large proportion of the active parts of sunlight. The doubled piece of calico, 2a, is seen to be as effective a shade as the white drill but both these are more translucent than the pale blue cloth XI. Even with these the proportion of ultra-violet rays that penetrate may be practically harmless to the human skin, while they must be altogether negligible from physiological standpoints with the more opaque cloths that have allowed only small effects on the photographic paper during the longer exposures. With the aid of some kind of optical wedge, definite comparisons of translucency could be obtained but the means were not available and the results of simpler methods seem to be enough for our immediate purpose.

There appears to be no doubt that radiations of the shorter wave lengths are responsible for sun burning, since very similar effects can be produced by artificial sources of ultra-violet light. In comparing such artificial effects it is to be noted that much shorter wave lengths may be involved, than those of the radiations which reach us from the sun. Meithe and Lehmann have shown that these are cut off, regardless of terrestrial altitude, at  $0.291\mu$ , though Dember, with more sensitive apparatus, recorded effects at  $0.280\mu$ . The results previously obtained by Cornu suggest that the shortest effective wave lengths reaching us from the sun are in the vicinity of  $0.295\mu$ , and Lyman<sup>1</sup> remarks that ever since quartz apparatus and the photographic plate have been used in the study of the solar spectrum, it has been found to become suddenly weakened near  $0.3\mu$ . Until experiment has proved the contrary it appears justifiable to neglect the physiological action of solar rays of wave lengths shorter than  $0.295\mu$  and possibly those between this and  $0.3\mu$  are

<sup>1</sup> Lyman (1914), *Spectroscopy of the Extreme Ultra-Violet*, London, p. 18.



too attenuated to be of much importance. This brings us to possible fallacies in connection with the experiment with photographic paper and the translucency of cloths. The cloths were covered with a sheet of glass about 1.1 mm. thick which is certain to have absorbed some of the shortest rays, nor can I state that the gelatino-citro-chloride emulsion of the photographic paper was fully sensitive to the shortest wave lengths of sunlight. The limitations of such paper are doubtless well known, consequently it is unnecessary to discuss them. To obtain evidence on the transparency of the glass plate a piece of the paper was partly shaded by it and exposed to full sunlight for several minutes. On the print the difference in tint between the shaded and unshaded parts is so slight that it is difficult to say with certainty that the unshaded part is darker. This tends to show that the glass has no marked absorption in the region of the solar rays that chiefly affects the emulsion, which presumably, in common with other photographic preparations of gelatine and silver salts, is sensitive to a considerable range of rays in the ultra-violet. In any case it appears reasonable to conclude that cloths that have formed effective screens to part of the longer ultra-violet waves will prove quite as efficient in the region of the shortest wave lengths included in sunlight.

Turning now to the practical effects of the cloths, my skin is extremely sensitive to sunburn, and I have found by experience that white calico, 2, which proved the most translucent of the cloths tested, is an efficient protection so that my skin does not become discoloured even after prolonged exposure beneath it. For example, in the course of journeys involving many days riding and walking, a shirt of a single thickness of such cloth has proved ample covering even through the middle hours of the day. There are many facts that suggest that the harmfulness of the ultra-violet rays of sunlight in the tropics have been much over-rated, and among them may be noted the conditions prevailing in Alpine resorts where, possibly owing to the altitude (Langley), but perhaps mainly due to the efficiency of snow<sup>1</sup> in reflecting rays of the shorter wave lengths, there are exhibited ordinary effects of ultra-violet light such as extreme sun-burning and conjunctivitis of the eyes; yet it appears that no case of "sunstroke" has been recorded from these cold regions. Other illustrations can be drawn from elevated districts within the tropics such as the plateaux of Abyssinia, the higher parts of East Africa and South America, where, so long as the temperature is cool, life seems to be pursued with little attention to the violet and ultra-violet rays which must be at least as powerful as in the hotter, low-lying parts where protection against them has been so strongly upheld. It remains to be remarked that in the hottest parts of the tropics where conditions border on the limits capable of being endured by man every additional disturbing factor is of importance, and among these is the sensation of glare which certainly promotes headaches and may predispose to other disorders even though the light to which it is

<sup>1</sup> J. V. Kowalski, *Nature*, LXXXII. 144.

due is less than that withstood without complaint in neighbouring regions of considerable altitude. For such reasons, hats should shade the eyes.

The height of the sun has an important influence and though the light appears bright it seems to have no burning effect on the skin for about two hours after it has risen, or until it has reached an altitude of nearly  $30^{\circ}$ . The onset of the burning is then rapid, as if the atmosphere has a steep absorption curve for the rays which cause the effect. It then becomes suddenly obvious that skin hitherto exposed must be protected, the shirt sleeves rolled down for instance, if a painful burn is to be avoided. Doubtless the active rays are cut off when the sun falls below a certain height in the afternoon but the transition is more difficult to observe, suffice it that devotees of outdoor sports, such as are usually limited to the last hours of sunlight, do not appear to show marked sun-burning. There is a peculiarity connected with sun-burning that still remains to be explained, though a thin covering is enough to protect from it yet it does not seem essential that the sun rays should act directly. The brim of any good sun helmet is wide enough to keep the face completely in shadow yet the face becomes burnt right up to the point where the forehead comes in contact with the hat. The under side of the chin which can hardly ever be exposed to direct sunlight also becomes burnt, and thus it must be concluded that a proportion of the burning rays is capable of reflexion even from surfaces of dark soil such as prevail over large tracts of the Egyptian Sudan. Wind certainly promotes sun-burning and it was considered whether the effect might be indirect, the rays acting in some way on the air which then affected the skin but the protection afforded by a thin covering points to a simple action of rays whether these are direct or reflected.

#### GENERAL DISCUSSION.

##### HISTORICAL.

In this part of Central Africa it is still difficult to find the literature necessary to complete a full discussion of the subject of this paper, and it is hoped that this is enough excuse for omission to refer to the work of others, if such exists, covering the same or cognate ground. If such work exists, it does not seem to have gained the consideration that it deserves, since the aspects treated in the present paper have been largely overlooked in several of the standard works and introductory manuals dealing with health in the tropics.

The classical experiment on the subject was done by Benjamin Franklin about the close of the eighteenth century. He described it in a letter addressed to Mary Stevenson, which is quoted by John Tyndall<sup>1</sup> as a preface to a chapter on radiant heat. Franklin took a number of different coloured pieces of cloth and laid them on snow in sunlight. In due course he observed that

<sup>1</sup> Tyndall, J. (1871), *Fragments of Science*, 3rd ed., London, p. 220. Franklin's letter has been omitted in some of the later editions.



the black, having been warmed most by the sun, had sunk deepest and was no longer within the stroke of the sun's rays. The dark blue had sunk almost as deep as the black, the lighter blue not quite as much, and the other colours in proportion as they were lighter, while the white remained on the surface. He proceeds to comment on the experiment: "What signifies philosophy that does not apply to some use," and points out that black cloths are not so fit to wear in a hot sunny climate or season. That soldiers' and sailors' uniforms in the tropics should be of white but that ladies' summer hats should be lined with black so as not to reverberate on their faces the rays that are reflected up from the ground. He pointed out also that fruit walls, being blackened, receive so much heat from the sun as to continue warm in some degree through the night.

Leslie and Melloni may be mentioned as among those who carried on extensive researches, during the early part of the nineteenth century, on the absorptive and emissive powers of various substances. Very full accounts of this work are to be found in some of the older text-books and a brief reference is all that is required here. They studied the emissivities of different pigments on the surfaces of vessels containing boiling water, and Melloni observed the different amounts of absorption of the radiations emitted by such sources as the Locatelli lamp, incandescent platinum, copper at  $400^{\circ}\text{C}$ . and copper at  $100^{\circ}\text{C}$ . The field was subsequently entered by Tyndall, who pointed out that the earlier investigators had neglected the absorptive properties of the gum which they had used in the application of their pigments. All these researches dealt with comparatively low temperature sources which emit radiations of long wave lengths, and consequently the results obtained cannot be compared with those due to solar radiation, which has its maximum within the range of the visible spectrum. With these longer wave lengths, Tyndall found it possible to show that a black looking substance, iodine, might reflect heat, while a white one, alum, absorbed it, becoming very hot and, with the results of such an experiment, he turned to one of his audiences with the remark that "this simple result abolishes a hundred conclusions hastily drawn from the experiment of Franklin<sup>1</sup>." Tyndall was under the impression that, even in the case of the sun, the bulk of the radiations consisted of invisible calorific rays, and he regarded the snow as at least as good an absorber of these long rays as the white cloth which did not sink into its surface. The sinking of the black cloth he explained by pointing out that, besides absorbing the heat rays just as the white one did, it also added the absorption of the whole of the luminous rays. The weight of Tyndall's authority and the wide popularity of his writings are doubtless the principal reasons why the absorptive properties of cloths and structural materials has received so little attention since.

The fallacy in Tyndall's position is that the maximum energy in the solar spectrum at the earth's surface is well within the limits of the visible

<sup>1</sup> *Fragments of Science*, 6th ed., London, 1879, i. 88.

spectrum, even under various conditions of terrestrial altitude and the sun's height. The relative intensity of solar radiation is dealt with on the basis of Smithsonian physical tables by Messrs J. D. Edwards and M. B. Long<sup>1</sup>, and their curves show that the proportion falls rapidly at the beginning of the infra-red, so that it is already small at  $1.0\mu$  and becomes practically negligible beyond  $2.0\mu$ . Langley traced it as far as  $5.5\mu$ .

In recent years the absorptive properties of pigments have come into prominence in connection with the painting of oil reservoirs and balloon fabrics. Experiments in regard to the former were described before the Pennsylvania State Association of Master-painters<sup>2</sup>. A caron arc was used as the radiant source so that the results are not comparable with those to be obtained with solar rays. The figures, for instance, yielded by tin and aluminium plates, were comparable with those for white, and this was certainly due to the much greater proportion of longer waves in the spectrum of incandescent carbon. The properties of balloon fabrics have been described by Messrs J. D. Edwards and M. B. Long in a very important paper<sup>3</sup>. They dealt with the reflexion, transmission and absorptive properties, and used the rays from a tungsten lamp, after passage through a 2 per cent. solution of cupric chloride, which they estimated had a maximum near  $0.6\mu$  as compared with  $0.5\mu$  for the sun's rays at the earth's surface. Some measurements with sunlight were also made and, while they differ, yet they show that valuable conclusions could be drawn from those made with the artificial light.

The temperature experiments described in the present paper are distinguished from most of the former ones in that they have been made by solar radiation and, moreover, they have been carried out in a part of the tropics where thermal environment has a very direct bearing on man's comfort.

#### THE PRESENT EXPERIMENTS.

At the outset it is to be noted that the experiments have been carried out either in Halfa, 125 m. above sea level, or in Khartoum, 383 m. above sea level. These altitudes are not great but the relative humidity at 2 p.m. at the seasons during which the temperature observations were made averages from 15 per cent. to 20 per cent. Such figures show that, at any rate, the surface layers must be more transparent than may be the case in humid parts of the tropics. The small humidity does not apply to the upper layers as may be seen sometimes by the existence of high clouds often moving in a direction quite different from that of the surface wind. The occasional presence of such clouds suggests that, at other times, there are masses of humid air which are not at saturation point and remain invisible to the eye. Since the sunlight has been filtered through them, little difference is to be expected in the character of the radiation as compared with that in other regions. Inequalities

<sup>1</sup> *Effect of Solar Radiation upon Balloons*, Bureau of Standards, Washington, 1919, p. 5.

<sup>2</sup> *Scientific American*, cxvi. 151, 1917.

<sup>3</sup> *Op. cit.*



of this kind appear to be a likely cause of the irregularities that occur in some of the temperature curves. The most striking instance is the fall recorded at midday during an experiment at Halfa on May 15, 1916. Smaller irregularities occur in nearly all the results given by painted flasks, while the cloths have yielded much smoother records. These smaller irregularities may be partly due to local air conditions in varying the amount of loss due to convection. Such matters are only incidents in the investigation and whatever the causes may be they do not affect the main thesis.

The temperatures recorded under the various cloths and paints merely represent the points at which equilibrium was established between the gain due to absorption and the losses due to emission and convection. Immediately this is recognised, it is obvious that the rate of heat absorption for the hotter cloths and paints is very much higher compared with the cooler ones, and is not indicated by the simple difference in temperature. According to Stefan's law the radiation is proportional to the fourth power of the absolute temperature and in addition there are the losses due to convection.

Cloths offer simpler cases than the paints since the losses are to a less extent due to convection. In looking at the relationships of the colours to temperatures, we have hitherto been looking at only one side of the equation, and it becomes of importance to consider the emissive properties of the various materials. The emission can only occur in the form of long waves such as issue from bodies at  $100^{\circ}\text{C}$ . or less and, so long as the substances behave as "black bodies" in this part of the spectrum, we are justified in considering the rate of absorption of solar rays as the principal factor in the temperature attained on exposure to the sun. Aitken<sup>1</sup> studied the radiating powers of cloths at temperatures in the neighbourhood of freezing point and found that there was no appreciable difference between black and white fabrics, nor did the experiments show any difference in radiating powers of cotton, wool or paint. He experimented with a number of substances and remarks that sulphur was one of the few substances which radiate less heat than a black surface. The emissive powers of various substances at  $100^{\circ}\text{C}$ . were compared by Leslie, who found that white lead radiated as efficiently as lamp-black and writing paper almost equally well. Tyndall pointed out that some of Leslie's conclusions were vitiated by the medium which he had used in the application of powders and that both gum and gelatine are good radiators. The influence of the medium, therefore, is to be borne in mind in considering the results yielded by our painted flasks. With the exception of the whitewash some medium was involved in the application of all the paints tested and quite possibly had as great an influence on the emissive properties as the pigments. The low emissive properties of metals at such temperatures, particularly from their polished surfaces, are well known. Aitken experimented with a polished piece of tin at temperatures near freezing point and his results bore out those of previous investigators in showing that

<sup>1</sup> John Aitken, *Trans. Roy. Soc. Edin.* xxxiii. 36 *et seq.*

there was little loss by radiation. Coblentz<sup>1</sup> records that they all have high reflecting properties for waves longer than  $3\mu$ , but the absorption becomes considerable in the visible part of the spectrum. Recent experiments<sup>2</sup> on radiation up to temperatures of  $200^{\circ}\text{C}$ . have generally borne out Leslie's and Tyndall's results, and it is stated that a coat of almost any kind of paint, regardless of colour, gives from 80 per cent. to 90 per cent. of the radiation of a black body. Aluminium paint gives only 45–55 per cent. of the radiation, but this depends on the nature of the vehicle, so that what appears the same to the eye may differ considerably in radiation.

In order to obtain some direct evidence on emissive properties of the painted flasks, measured quantities of water from a boiling kettle were poured into four of them. They were placed on the mat-covered wooden floor of a room where the air was kept as still as possible and the water was stirred and the temperatures read at intervals with the following results:

	9.35 p.m.	10.21 p.m.	11.30 p.m.
Whitewash	86.0	60.6	45.5
Black paint	86.3	61.7	46.5
White enamel	88.0	63.1	47.0
Dark green	86.8	64.2	48.2

These figures tend to show that there were very small differences in the rates of cooling and such as there were are possibly well within the limits of experimental error.

The properties of metallic surfaces are involved in the cases of some flasks included in the combined experiment of June 14, 1918. The plain tin attained 57.2 per cent. of the difference between the black and white standards. Two flasks were painted half black and the other halves were plain tin and whitewashed respectively. During the experiment the black halves were kept facing the sun so that the absorption occurred on that face. The temperature of the flask with the nether half whitewashed proved cooler than that with the half plain tin. Since the black surfaces must have absorbed approximately equal quantities of heat, the difference of temperature was due to the greater emissivity of the whitewash, which is what was to be expected from the known properties of the materials. The comparatively high temperature of the tin is due to the poor emissivity for the longer waves, allowing relatively high temperature to be attained before equilibrium is established with the rate of absorption from the solar rays, even though it may not absorb as efficiently as some paints. Fabry<sup>3</sup> has pictured an extreme case of this kind by supposing a body to have selective absorption for a wave length of  $0.4\mu$  and this, he states, if situated in space at the same distance as the earth from the sun, would attain a temperature of  $198^{\circ}\text{C}$ . before equilibrium with the emission was reached.

<sup>1</sup> *Investigations of Infra Red Spectra*, Part IV, Washington, 1906, p. 98.

<sup>2</sup> J. A. Harker, *Nature*, cii. 324.

<sup>3</sup> *Astrophysical Journal*, xiv. 269.



The aluminium paint presents the appearance of a metallic surface but is actually different owing to the varnish medium in which the metallic pigment is embedded. The absorption is doubtless mainly due to the metal, and the emissivity may be controlled by the medium so resulting in a temperature in sunlight that is about 55 per cent. of the difference between the black and white standards. The half black and half white flask included in the combined experiment with its black side towards the sun gave temperatures appreciably lower than the black standard and this, alone, might suggest that the whitewash has a greater emissivity at such temperatures than the lamp-black and varnish. This is not borne out by the experimental data referred to above, though lamp-black is known to be transparent for the longer wave lengths and, therefore, is likely to have a correspondingly less emissivity.

The character of an ideal, cool, paint for exposed surfaces in a sunny tropical climate is one with the smallest absorption in the visible spectrum, and the highest possible emission in the longer wave lengths. The radiant heat from the inner surface might be lessened further if that could be made of bright metal or perhaps coated with aluminium paint which appears to have a smaller emissivity for the longer wave lengths than other paints. In practice, for outer surfaces, plain whitewash approaches nearest the ideal and white oxide paints are almost as good as whitewash. These give comparatively low temperatures so that the emissivity of the inner surface becomes of little consequence.

In regard to cloths, the emissivities for the longer rays appear to be much alike and the controlling factor is the absorptive power for sunlight. Here also white, is the coolest colour and the experiments show the nature of the thermal burden involved in wearing khaki or black cloths in the tropical sun.

The experiments have been mainly concerned with ordinary fabrics or paints on their supports. In practice, thick materials and massive structures are often involved. The ordinary pith helmet may be cited as an example of a thick material which may be absorptive according to its colour, but owing to the low conductivity of the pith, the temperature of the inside of the helmet is almost completely controlled by that of the freely circulating air. At the suggestion of Mr H. E. Hurst, the influence of white layers under khaki was tried, with a view to obtaining evidence on the thermal conditions ruling when a khaki coat is worn over a white shirt. It was found that the intervening white layer made practically no difference to the temperature, which therefore depends on the colour exposed since there is no free circulation of air to affect the temperature by convection. Certain roofing materials afford other examples, and in these, sometimes owing to the less free circulation of the air below and the continued exposure on a sunny day, the warmth of a hot outer colour may sometimes gradually penetrate. If coolness is desired the roof should be white. A massive structure may be exemplified by the brick wall of a house. The amount of heat it takes up depends on the

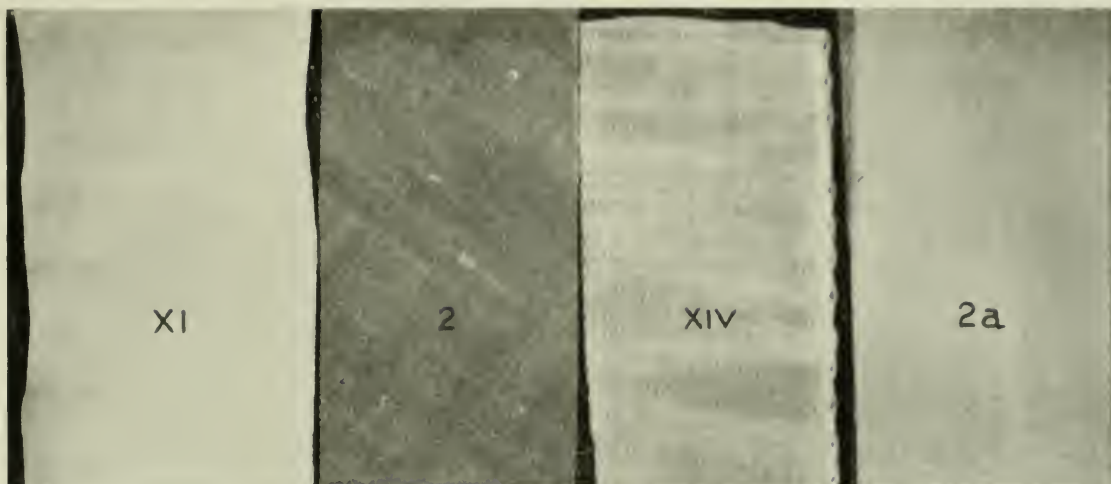
surface it presents to the sun. If this is dark and in sunlight for many hours during the day, a great deal of heat will be absorbed and the wall will be found to remain hot for a long time. A white wall absorbs less heat, consequently has less to lose and must be correspondingly cooler.

This paper has presented the results of a number of experiments on the temperatures reached by different coloured cloths and paints exposed to sunlight, and reference to the figures, whatever the explanations may be, shows the general coolness of white as compared with other colours and the very striking differences that may occur. Some attention has been paid to texture of cloths. Translucency has also been studied and it has been shown that many ordinary cloths are very opaque to actinic rays. Even fairly thin, white cloth appears to offer all the protection necessary to preserve tender skin from ordinary physiological effects of the shorter wave lengths of solar light. When some coloured cloths are worn, a pad of non-conducting material may be desirable to protect such parts as the spine from actual heat. It cannot have any appreciable influence as far as the violet rays are concerned. A white paint is advantageous for exposed wood work, since the timber is not subject to the thermal range that must occur with colours. Apart from temperature there is another advantage in white clothes, for I have often observed that noxious flies, such as the tse-tse, are not attracted by white clothing, consequently the wearer is less liable to attack. The mosquito seeks dark places to roost, and the more light that can be propagated by white or light colours, the less will be the attraction for these insects.

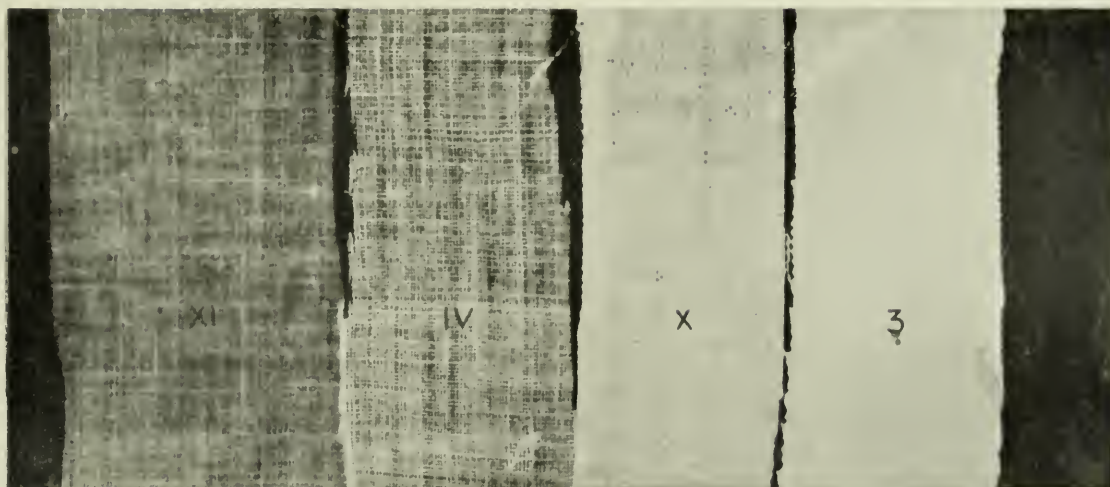
In conclusion, I record my thanks to the Public Works and Railways Departments of the Sudan Government for help they have given and the interest taken in the results. My thanks are due to many for much kindly discussion, and in particular to Mr H. E. Hurst, who lent me the evaporimeters.



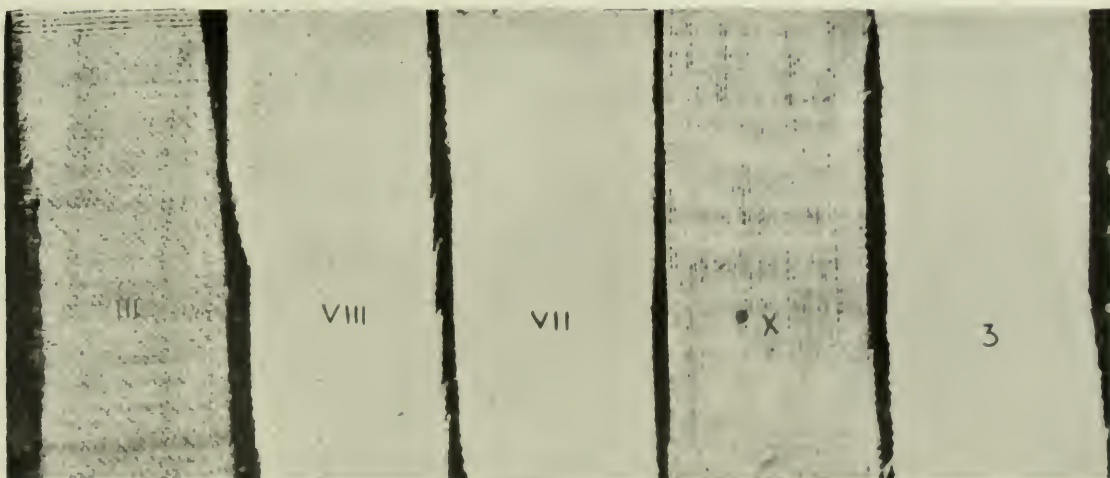
276<sup>2</sup>



SET 1. 10.52—10.57 a.m. 14. vi. 18.



SET 2. 12.42—1.12 p.m. 14. vi. 18.



SET 3. 11.7 a.m.—12.7 p.m. 4. x. 18.

TRANSLUCENCY OF CLOTHS.





## THE WASSERMANN TEST IN PATIENTS AFFECTED WITH MALARIA IN THE TROPICS.

BY DR F. H. HEHEWERTH AND W. A. KOP.

(*Central Military Medical Laboratory, Java.*)

### INTRODUCTION.

WITH regard to the influence of malarial infection on the Wassermann test some controversy prevails:

Wassermann and Lange (1913) refer to Meyer and Bonfiglio who, working in Italy, obtained a positive reaction in 80 per cent. of the cases where the blood contained parasites; in some of their cases positive reactions were obtainable for several months after the disappearance of fever and parasites. For this reason Wassermann and Lange advise to put every case, coming from a malaria-infected country and showing a positive reaction, under quinine treatment and to repeat the test after a lapse of six weeks.

Schoo (1910), Zschucke (1913), de Haan (1913), Meyerstein (1917) and others also obtained a considerable number of positive reactions. Schoo obtained 22 positives out of 38 cases; in several of his cases the blood was free from parasites and there had been no fever for many days. Zschucke, using the Brendel-Müller modified method, obtained 14 positives out of 17 fresh cases.

De Haan experimented at Batavia, Java, on the same class of patients as ours but used Wassermann's original method. His investigations being founded on a sound basis, it is to be regretted that his work was curtailed. He sought especially to discover how long the test would continue positive. With the exception of two cases, his examinations were only continued for three weeks, consequently his results were unsatisfactory. He obtained 63 positive results in a series of 153 cases (40 Europeans and 113 natives). Of the Europeans nine were positive and 31 negative. Of the natives 51 were positive and 62 negative. Among the 60 positives were 29 cases wherein syphilis could be excluded, these latter were examined at regular intervals for some time. Of six cases of benign tertian only two were still positive after 17 days. Of 23 cases of subtertian fever eight were negative and 15 were still positive after 24 days. Of the latter only two were examined again after 40 and 49 days and still found to be positive.

Meyerstein (1917) does not state the number of cases he examined, they were mostly cases of benign tertian and he concludes that: (1) the strongest reaction is found on the fifth to eighth day, 70 to 80 per cent. of malaria

cases being positive; (2) a positive result after the tenth day is very rare; (3) with quinine treatment the positive result disappears; (4) disappearance of the positive result during quinine treatment does not mean definite cure of the malaria, but cases which continue positive (syphilis being excluded) must be considered as uncured.

Sutherland and Mitra (1915) consider malaria to have little influence on the Wassermann test. They examined 32 cases of benign tertian and 18 of subtertian fever. Though they only took blood for the test during the fever and in nine cases obtained a positive result, they come to the perhaps somewhat rash conclusion that a positive result occurs sometimes while the fever lasts, but that in chronic cases no influence is exerted upon the Wassermann reaction. It would be sufficient to wait a week after the disappearance of the plasmodia from the blood, after which the Wassermann test would be reliable.

de Jong (1919) came to a similar conclusion. We cannot, however, discuss his results, since we have only had access to a review of his paper.

#### OUR INVESTIGATIONS.

None of the papers on the Wassermann test in malaria mention a quantitative result of the test and none give sufficient data regarding the time during which a positive result persisted.

Therefore we carried out our tests (1) during the fever while there were plasmodia in the blood; (2) after one or two weeks of a good quinine-arsenic treatment; (3) at regular intervals until negative results were obtained; (4) two complete tests were carried out in each case, one with heated (56° C.) and one with unheated serum.

As described in a previously published paper (1919) we use alcoholic extract of human heart. The strength of the complement is determined and a complement-unit established. The test is carried out with one, two, four and eight times the complement-unit. Human serum is used in the same amount as the extract. Of every case we recorded the race to which the patient belonged, the kind of plasmodium, the type of fever, if quinine was used while the blood was taken, and data bearing on possible syphilis or yaws and relapses of malaria.

Possibly syphilitic cases were only recorded when the Wassermann reaction became negative. Here we took for granted that a positive result in syphilis will not become negative in a short time without antisiphilitic treatment. We only considered cases as free from syphilitic infection after thorough investigation; even slightly dubious cases were always excluded. All cases were treated by one of us in the wards of the Tropical School. Apart from natives, our records relate to but three Europeans (syphilitics being barred).

As most of the tests had to be made after the men left hospital we only tested soldiers because we could call them up again when required. Owing to considerable shifting about of men constituting the Batavia garrison we



lost many cases before their test became negative. In such cases we used the decrease of strength in the reaction as an index upon which, in part, to base our conclusions.

**Europeans** (3 cases). With heated serum all gave negative results. With unheated serum all gave positive Wassermann reactions which became negative after two, three and eight weeks.

**Natives** (41 cases). With heated serum 21 were positive and 20 negative. With unheated serum 38 positive and three negative. Only three natives yielded a completely negative result with the Wassermann test.

*Strength of Wassermann test in malaria.* With heated serum, two cases reached our final limit (+ 8) (Nos. 31 and 40).

With unheated serum, we obtained higher values than with heated, as we generally do with this method.

*Period during which a positive result persists.* With heated serum strong positive reactions persisted for a considerable time (Nos. 24, 26, 27, 28, 29, 31, 34) often more than three months. Mostly the strength of the reaction decreased (Nos. 27, 29, 31, 34).

With unheated serum, often the lapse of five to six months is insufficient to yield a negative result (Nos. 19, 23, 25, 27, 29).

*Benign tertian and quartan fever.* Out of 12 cases tested with heated serum four gave positive and eight negative results. Of the positive cases three were negative within 14 days, and one was still positive.

With unheated serum all 12 were positive, eight became negative and four remained positive.

*Subtertian fever.* With heated serum, out of 31 cases 17 were positive and 14 negative. Of the positive cases, four became negative after two, seven, six and eight weeks respectively. Many of them were examined not longer than two weeks after the fever, hence there might have been more negatives if we had been able to examine them again. With unheated serum only three were negative and 28 positive. Here too it must be considered that many could not be examined after two weeks had lapsed from when the fever ceased.

The degree of splenic enlargement was without influence on the strength of the reaction. Subtertian fever seems to give stronger and more persistent positive results than benign tertian and quartan fever. Europeans do not seem to give a positive result so frequently as natives, at least not with heated serum; this may be due to the frequent chronicity of malaria in natives. We are inclined to believe that the long persisting positive Wassermann reaction is a result of defective treatment.

All cases got 15 grains of quinine hydrochl. twice a day with liquor Fowleri 5 to 15 minims three times a day for 14 days. After that period they left the hospital and were supposed to get 15 grains of quinine twice a week for three months, but it is very doubtful that this course of treatment was actually pursued. Better treatment would perhaps have yielded more negatives and in a shorter time.

The great number of positive results obtained with Wassermann tests in natives naturally arouses suspicion that hidden endemic syphilis, acquired and congenital, may have existed amongst them. Now (1) a tremendous degree of infection would have to prevail if out of specially picked persons, without any sign or history of syphilis, 50 per cent. yielded positive results for syphilis by the ordinary Wassermann test; (2) we (1919) carried out the same test on natives of the same kind, *i.e.* healthy subjects that were, as far as we could ascertain, free from syphilis and malaria; these subjects were used to test our Wassermann with unheated serum in our search for non-specific reactions. In 21 native soldiers, using the ordinary Wassermann test with heated serum, *all* yielded negative results. With unheated serum nine tests were negative and 12 positive, the strength of the positive cases not exceeding + 1. We next tried other natives and obtained similar results. Out of 35 cases 34 were negative and one yielded a slightly positive reaction with heated serum. With unheated serum 21 were negative and 14 gave slightly positive results.

Syphilis is rather common in Java and the results mentioned are of course not to be considered as showing the degree of infection, they merely afford evidence that our results with malaria are not impugned by the presence of hidden syphilis. When we compare the results obtained with malaria, where 50 per cent. were positive (heated) and 95 per cent. positive (unheated) with mostly strong reactions up to + 8, while with the non-malarious natives none was positive with heated and only slightly positives occurred with unheated serum, anybody can see that it is the malaria and not hidden syphilis that is giving the difference.

#### CONCLUSIONS.

As a result of our investigations, in connection with those of de Haan, we feel entitled to draw the following conclusions:

(1) In many cases malarial infection causes a positive reaction to be obtained with the Wassermann test; 50 per cent. positives were obtained in natives whereas in Europeans no rate could be determined because of the small number of cases available.

(2) The reaction may even attain a maximum.

(3) A positive reaction may persist for three to six months; it may also persist as long in thoroughly treated patients but the reactions obtained in such cases are as a rule rather weaker.

(4) The positive reaction vanishes as a rule under quinine treatment.

(5) The Wassermann test with unheated serum almost always gives a positive result in both natives and Europeans affected with malaria.

(6) The Wassermann test with unheated serum yields positive results for longer periods and gives stronger reactions in natives than in Europeans.

(7) The difference which we (1919) found between Europeans and natives when we applied the Wassermann test with *unheated* serum (*viz.* that, when



*Table recording Wassermann Tests carried out on 44 persons.*

Number	Europeans	Natives	Syphilis	Framboesia	Kind of parasites			1st W. R.		2nd		3rd		4th		5th		6th		7th	
					Tertiana	Quartana	Tropica	Date	Inactivated	Not inactivated	Date	Inactivated	Not inactivated	Date	Inactivated	Not inactivated	Date	Inactivated	Not inactivated	Date	Inactivated
1	x	.	1915	-	+	+	25. iii.	-	+	+	26. iii.	-	+	+	+	15. iv.	-	+	+	+	7th
2	x	.	1915	-	+	+	23. iv.	-	-	-	6. v.	-	-	-	-	1. viii.	-	-	-	-	6th
3	x	.	1915	-	+	+	20. v.	-	-	-	3. vi.	-	-	-	-	1. viii.	-	-	-	-	6th
4	.	x	1915	-	+	+	20. v.	-	-	-	3. vi.	-	-	-	-	1. viii.	-	-	-	-	6th
5	.	x	1915	-	+	+	20. v.	-	-	-	8. vii.	-	-	-	-	1. viii.	-	-	-	-	6th
6	.	x	1915	-	+	+	24. vi.	-	-	-	8. vii.	-	-	-	-	1. viii.	-	-	-	-	6th
7	.	x	1915	-	+	+	24. vi.	-	-	-	8. vii.	-	-	-	-	1. viii.	-	-	-	-	6th
8	.	x	1915	-	+	+	25. ii.	-	-	-	4. vii.	-	-	-	-	12. iii.	-	-	-	-	6th
9	.	x	1915	-	+	+	25. ii.	-	-	-	4. vii.	-	-	-	-	1. viii.	-	-	-	-	6th
10	.	x	1915	-	+	+	3. vi.	-	-	-	17. vi.	-	-	-	-	1. viii.	-	-	-	-	6th
11	.	x	1915	-	+	+	17. vi.	-	-	-	8. vii.	-	-	-	-	1. viii.	-	-	-	-	6th
12	.	x	1915	-	+	+	15. vii.	-	-	-	1. viii.	-	-	-	-	30. ix.	-	-	-	-	6th
13	.	x	1917	-	+	+	23. iv.	-	-	-	6. v.	-	-	-	-	13. v.	-	-	-	-	6th
14	.	x	1917	-	+	+	25. ii.	-	-	-	4. iii.	-	-	-	-	9. iv.	-	-	-	-	6th
15	.	x	1917	-	+	+	4. iii.	-	-	-	11. iii.	-	-	-	-	1. iv.	-	-	-	-	6th
16	.	x	1917	-	+	+	4. iii.	-	-	-	12. iii.	-	-	-	-	26. iii.	-	-	-	-	6th
17	.	x	1917	-	+	+	26. iii.	-	-	-	15. iv.	-	-	-	-	23. iv.	-	-	-	-	6th
18	.	x	1917	-	+	+	26. iii.	-	-	-	6. v.	-	-	-	-	1. viii.	-	-	-	-	6th
19	.	x	1917	-	+	+	26. iii.	-	-	-	6. v.	-	-	-	-	30. ix.	-	-	-	-	6th
20	.	x	1917	-	+	+	26. iii.	-	-	-	7. iv.	-	-	-	-	6. v.	-	-	-	-	6th
21	.	x	1917	-	+	+	26. v.	-	-	-	10. vi.	-	-	-	-	1. viii.	-	-	-	-	6th
22	.	x	1917	-	+	+	17. vi.	-	-	-	8. vii.	-	-	-	-	1. viii.	-	-	-	-	6th
23	.	x	1917	-	+	+	25. ii.	-	-	-	26. viii.	-	-	-	-	1. iv.	-	-	-	-	6th
24	.	x	1917	-	+	+	25. ii.	-	-	-	11. iii.	-	-	-	-	12. iii.	-	-	-	-	6th
25	.	x	1917	-	+	+	25. ii.	-	-	-	4. iii.	-	-	-	-	26. iii.	-	-	-	-	6th
26	.	x	1917	-	+	+	4. iii.	-	-	-	11. iii.	-	-	-	-	26. iii.	-	-	-	-	6th
27	.	x	1917	-	+	+	4. iii.	-	-	-	12. iii.	-	-	-	-	26. iii.	-	-	-	-	6th
28	.	x	1917	-	+	+	4. iii.	-	-	-	19. iii.	-	-	-	-	15. iv.	-	-	-	-	6th
29	.	x	1917	-	+	+	4. iii.	-	-	-	12. iii.	-	-	-	-	26. iii.	-	-	-	-	6th
30	.	x	1917	-	+	+	6. iv.	-	-	-	8. iv.	-	-	-	-	1. viii.	-	-	-	-	6th
31	.	x	1917	-	+	+	28. v.	-	-	-	10. vi.	-	-	-	-	30. ix.	-	-	-	-	6th
32	.	x	1917	-	+	+	3. vi.	-	-	-	10. vi.	-	-	-	-	30. ix.	-	-	-	-	6th
33	.	x	1917	-	+	+	3. vi.	-	-	-	26. vi.	-	-	-	-	20. viii.	-	-	-	-	6th
34	.	x	1917	-	+	+	10. vi.	-	-	-	26. iv.	-	-	-	-	30. ix.	-	-	-	-	6th
35	.	x	1917	-	+	+	17. vi.	-	-	-	1. vii.	-	-	-	-	30. ix.	-	-	-	-	6th
36	.	x	1917	-	+	+	8. vii.	-	-	-	22. vii.	-	-	-	-	20. viii.	-	-	-	-	6th
37	.	x	1917	-	+	+	15. vii.	-	-	-	1. viii.	-	-	-	-	1. viii.	-	-	-	-	6th
38	.	x	1917	-	+	+	15. vii.	-	-	-	22. vii.	-	-	-	-	30. ix.	-	-	-	-	6th
39	.	x	1917	-	+	+	22. vii.	-	-	-	1. viii.	-	-	-	-	30. ix.	-	-	-	-	6th
40	.	x	1917	-	+	+	22. vii.	-	-	-	1. viii.	-	-	-	-	30. ix.	-	-	-	-	6th
41	.	x	1917	-	+	+	24. vi.	-	-	-	5. viii.	-	-	-	-	30. ix.	-	-	-	-	6th
42	.	x	1917	-	+	+	24. vi.	-	-	-	8. vii.	-	-	-	-	30. ix.	-	-	-	-	6th
43	.	x	1917	-	+	+	15. vii.	-	-	-	22. vii.	-	-	-	-	30. ix.	-	-	-	-	6th
44	.	x	1917	-	+	+	15. vii.	-	-	-	22. vii.	-	-	-	-	30. ix.	-	-	-	-	6th

healthy and non-syphilitic, Europeans always give negative results, while natives sometimes give positive results) is probably a sequel of malaria.

For practical purposes we would establish the following rules:

I. *In a heavily infected malarial country a positive Wassermann reaction cannot be considered as due to syphilis if there is no special evidence in favour of syphilis.*

II. *If after a good quinine treatment a positive Wassermann reaction becomes negative or distinctly weaker, without antisyphilitic treatment, probably the positive Wassermann was a sequel of malaria.*

III. *Especially in natives a positive Wassermann reaction does not permit of conclusions without careful consideration; a positive result with unheated serum is in such cases almost useless, a negative result of course will be as valuable as elsewhere.*

IV. *In natives, a Wassermann test conducted with heated serum within three months from an attack of malaria is of little value for the diagnosis of syphilis. To consider a test made not more than a week after the attack as advised by Sutherland and Mitra is certainly wrong.*

#### REFERENCES.

- DE HAAN (1913). *Geneeskundig Tijdschrift v. Ned. Indië*, p. 737.  
 HEHEWERTH, F. H. en KOP, W. A. (1919). *Techniek en methodiek der Wassermannsche reactie in de tropen. Geneesk. Tijdschrift v. Ned. Indië*, p. 499.  
 DE JONG (1919). *Bulletin Institut Pasteur*, No. 8, Analyses.  
 MEYERSTEIN (1917). *München med. Wochenschr.* p. 366.  
 SCHOO (1910). *Nederlandsche Tijdschrift voor Geneeskunde*, p. 295.  
 SUTHERLAND and MITRA (1914). *Indian Journ. Med. Research*, II. 984.  
 WASSERMANN, A. VON and LANGE, C. (1913) in Kolle and Wassermann's *Handb. der pathog. Mikroorg.* VII. p. 1007.  
 ZSCHUCKE (1913). *Berlin klin. Wochenschr.* p. 1716.



CLINICAL INVESTIGATION OF XEROPHTHALMIA  
AND DYSTROPHY IN INFANTS AND YOUNG  
CHILDREN (*XEROPHTHALMIA ET DYSTROPHIA  
ALIPOGENETICA*).

By C. E. BLOCH, M.D.,

*Professor of Pediatrics in the University of Copenhagen.*

(With Plates II–IV and 5 Charts.)

*Preface by the Accessory Food Factors Committee (appointed jointly  
by the Lister Institute and the Medical Research Council).*

THE investigations of Professor Bloch upon xerophthalmia in infants and young children are of unusual interest when considered in conjunction with the experimental production of the disease in rats by a deficient diet. The history of Professor Bloch's researches is particularly instructive. The first cases of xerophthalmia in infants only came under his notice in the Children's Department of the State Hospital in Copenhagen, after they had been under (unsuccessful) treatment in the Ophthalmic Department of the same institution. It was noticed in many instances that the children showed signs of general malnutrition in greater or less degree and it was while giving attention to this aspect of his cases that Professor Bloch discovered the striking curative effect upon the eye disorder of giving a diet rich in fat, *e.g.* full milk and especially cod-liver oil. Later, on becoming acquainted with the experimental work upon xerophthalmia caused in rats by a deficiency of the "fat-soluble" accessory factor, he was able to devise a treatment consisting solely of addition to the diet of cod-liver oil, as was carried out in the eight cases in the Copenhagen Children's Home, described below.

Professor Bloch's work was published in Danish in 1917 and 1918 and was therefore not available for the majority of English readers. The Committee on Accessory Factors therefore undertook the publication in English of an abridged translation of two of the later papers, to which Professor Bloch has added a short account of his experience in 1918 and 1919. In the meantime the more important of these papers, but without photographs, has appeared in the *Jahrbuch für Kinderheilkunde* (1919), vol. LXXXIX.

The Committee desires in this place to record its thanks to Dr Thaysen and to Dr E. E. Atkin, by whom the English translations were made.

Xerophthalmia is considered a rare disease. Only amongst the negro slaves of Brazil and amongst the poorest and most ignorant inhabitants of Russia is it said to have been observed to any extent. The disease is generally described as follows: the first symptoms are dryness of the ocular conjunctiva, which becomes wrinkled and shrunken. Later on small yellowish white spots appear, as though the conjunctiva had been dotted with paraffin wax. At this stage the disease is termed xerosis conjunctivae. The dryness rapidly spreads over the whole conjunctiva and over the cornea, which becomes dull, uniformly hazy and insensitive. Later the cornea turns greyish and still later yellowish, until at last a more or less extensive necrosis of the cornea sets in, followed by ulceration (keratomalacia). The necrosis and ulceration may appear in the course of a few hours.

It is characteristic of the disease that there is hardly any reaction in spite of the far-reaching changes in the eye; lachrymation, photophobia and spasm of the eyelids are absent. In the beginning no injection or ulceration of the mucous membrane is seen, and no secretion takes place. A very striking contrast thus exists between the severe and mild types of the disease.

All authors agree that the keratomalacia is due to insufficient nutrition of the cornea, and that this again is a consequence of the disorganisation of the whole mechanism of nutrition. The disease therefore only occurs amongst children who have been ailing for a considerable time, and is often met with amongst infants who have been insufficiently nourished. Especially it is observed after prolonged diarrhoea, and debilitating diseases, such as typhoid fever, scarlatina, hereditary syphilis, etc.

My own material comprises 40 cases from 1912–1916 and 23 cases from 1917 onwards, all of which have occurred amongst children treated in the children's section of the State Hospital.

Of the 40 cases in 1912–1916 five showed xerosis alone; in the other 35 cases the cornea was attacked, 28 cases showing keratomalacia of both eyes and seven cases of one eye only. In many cases the necrosis of both corneae was complete. There was ulceration of the cornea in nearly all, several showing prolapse of the iris, and a few, extrusion of the lens. My material thus comprises mainly severe and serious cases of this disease.

The actual effect on the eye will not be discussed in further detail. There is only one point which I should like to draw attention to, because my cases exhibit certain aspects, which do not entirely agree with earlier observations. Only in three cases of xerosis and three cases of keratomalacia were the lesions slight. The eye condition in these cases was of minor importance compared with the other symptoms. Those associated with the children had hardly noticed that the eyes were abnormal. Only on examination at hospital were the eyes found to be affected and the disease must therefore have taken a very acute course. These cases are almost identical with those described by other authors. They all occurred in miserably emaciated children, only a few months old. In none of the cases did the keratomalacia reach its climax



with extensive ulcerations and gangrene. The children died from marasmus and its complications before this stage was reached.

In all the other cases examined by me there were marked lesions of the eyes, contrary to what had been observed before. In many cases the damage was extraordinarily extensive, and it was generally the irritation of the eyes that had induced the parents to seek medical advice. Lachrymation and photophobia were generally given as the first symptoms, but injection, ulceration and abundant discharge from the eye were also observed. In addition there was also spasm of the eyelids. Most cases thus showed both xerophthalmia and conjunctivitis. The course of the eye complaint could be followed by comparing the various patients, or the two eyes of one and the same patient; as the disease could often be found in different stages in the two eyes.

The first change obviously had been xerosis of the conjunctiva. The children felt that something was wrong and perhaps they could not see so well as before. They would therefore begin rubbing their eyes and the parents have often noticed this. This rubbing caused conjunctivitis, and its accompanying photophobia and discharge from the eyes. Gradually the discharge grew worse and this generally led the parents to seek medical advice.

In many cases the conjunctivitis appeared in addition to general catarrhal infection. The first sign that the child was unwell was a cold but the eyes continued to discharge after the cold had disappeared, and the condition grew worse.

In an isolated case the eye complaint appeared simultaneously with measles, and the doctor paid little attention to it. Only when the measles had disappeared and the eye trouble steadily grew worse did it become clear that it was due to something else. In the meantime, however, both corneae had been completely destroyed by necrosis.

In all these cases the eye lesion was only part of the disease. The constitution of the patients had been undermined, as was visible from the abnormal appearance of the children. The change was noticeable in various ways, from which three distinct types stand out, with practically every intermediate form. Two of these types are identical with the complaints described by Czerny and his co-workers under the name of "Mehlnährschaden." I term these two types: carbohydrate atrophy and carbohydrate dystrophy respectively. Pronounced cases of the third type were markedly different from the two others, as no atrophy, oedema or hypertonicity of the muscles were found, which are particularly characteristic of Types 1 (Pl. II, fig. 1) and 2 (Pl. II, fig. 2). Furthermore, these children had not had an excess of carbohydrate as had been the case with the others.

Type III. *Xerophthalmia and Dystrophia alipogenetica*.

This third group showed the least pronounced characteristics (Pl. II, fig. 3). At first sight the children seemed almost normal apart from the eye complaint, and one doubted whether any constitutional derangement existed. On closer examination, however, the children bore marked signs of a general breakdown in health. They were all very backward in their development and they were weak and thin, and markedly anaemic. When left to themselves they were apathetic and took no interest in their food. The older ones were hypersensitive to external impressions. They were as susceptible to secondary infections as the children showing marked dystrophy and atrophy.

The clinical information available clearly showed that these children had long been on an unsuitable and insufficient diet consisting of centrifuged milk, milk puddings made with this separated milk, and some of their parent's low diet, such as potatoes, bread, etc. The cause of the disease and particularly of the eye complaint was evidently not due to an excess or deficit of carbohydrate nor could it have been caused by the absence of protein, salts, or water-soluble vitamins as all of these substances were present in sufficient quantities in the children's food.

When I published my first paper it was unknown in this country that the American physiologists had obtained evidence of the presence of certain specific bodies in various fats.

None of my cases had received fresh milk for any length of time. They were all cured by cod liver oil and improved by fresh milk. I therefore attributed the disease to the absence of some sort of fat. This assumption was supported by observations made by Mori in Japan on a disease called hikan; which in many respects resembled my third type. The Japanese disease was considered to be due to the absence of fat in the food. My observations, however, have made it clear that the disease was not due to the absence of fat as such, but probably to the absence of certain bodies normally present in butter fat and in large quantities in cod liver oil. I therefore proclaimed that the disease was most probably due to "the absence of specific lipid bodies or their constituents" by which I meant to include all the possibilities. Since I considered that the stunted growth and general ill health as well as xerophthalmia were caused by lack of these substances, I called the disease *Dystrophia alipogenetica* to distinguish it from carbohydrate atrophy and I applied the same designation to xerophthalmia.

Since that time I have become acquainted with the experiments carried out on growth of young rats by McCollum and his co-workers and Osborne and Mendel.

From experiments carried out by McCollum and his collaborators, it appears that a fat free diet inhibits the growth of young rats and some time later their eyes become diseased. If this diet is continued the animals die. Later experiments have shown that these abnormalities were not due to the



absence of fats but to lack of a certain specific substance called "Fat soluble A," which is present in certain fats only. So far the substance has been found in milk and butter fat, egg yolk fat, and in the ether extract of kidneys and testes, in beef fat and in cod liver oil. As regards vegetable fats it has been found in small quantity in the wheat germ and in the ether extract of maize, but not in olive oil, cotton seed oil or almond oil. Pork fat or suet, in the condition in which these substances are sold in the markets, contained quantities inappreciable by their experiments.

The actual nature of the body is unknown. It has been found to be comparatively resistant and is not destroyed by boiling for a short time although it does not stand prolonged boiling or being heated to higher temperatures. It is also probable that it is rendered inactive by the various methods of purification and preservation resorted to, and this is possibly the reason why it has not been found in many of the fats sold in the shops.

Young rats fed on a diet containing fats of this latter type show the same pathological changes as are caused by the absence of fats. The first case where xerophthalmia was noticed in young rats occurred after a prolonged diet of protein-free milk, starch, and pork fat (Osborne and Mendel (1913-14)). The eye trouble is described as inflamed eyes gradually becoming purulent. When taken in time it was possible to cure the animals by feeding with butter fat or cod liver oil. Osborne and Mendel thus mention that a small quantity of cod liver oil given in lieu of pork fat is sufficient to restore the weight and cure the disease. The eyes rapidly become normal.

Thus it will be seen how close is the agreement between the experiments on animals and my previously published clinical observations. A prolonged unvaried diet, containing either no fats or no specific lipoid bodies (as far as children are concerned, lack of fresh milk), causes both children and rats to fall sick. In both cases the disease manifests itself first and foremost as a constitutional disturbance expressing itself as inhibited growth and loss of weight, a condition which I have termed dystrophy. In a number of cases eye trouble develops; at first the eyes are inflamed and later become purulent and necrotic. If the diet is persisted in, both children and animals die. In both cases the dystrophy and the eye lesions can be cured by administration of cod liver oil and butter fat.

During the year 1917 I again observed and treated numerous cases of these diseases. My new material has brought certain points to light and has confirmed and extended my already published contributions. Keeping in mind the results of the experiments on animals, it has been comparatively easy to deal with this additional material as knowledge has now been acquired concerning points which were previously difficult to interpret.

My cases of xerophthalmia in 1917 were 23 in number and exhibited a somewhat altered character, there being not only the extremely serious cases of keratomalacia, which predominated in earlier years, but also a large

number of milder cases. Of the 23 children, 11 had eye trouble for a few weeks before the real nature of the disease was discovered; these cases occurred amongst somewhat older children. As far as the eyes were concerned xerosis was the only lesion. Amongst my previous material there were no such cases.

Eight of the milder cases came from a Home for children in Copenhagen in the administration of which I take part. Six of these had been in the Home for more than a year and had developed well before the present illness so that they may be considered as originally healthy children. As the conditions giving rise to the disease are fairly well known in these instances, they may be considered as straightforward and simple as the animal experiments.

In this Children's Home there were 86 children separated into two sections, each with its own building. Section A comprised the infants and as far as possible, the delicate and sick children. The healthy children or those more than a year old were in Section B. Both sections were again divided into two subsections completely isolated from one another. Section B contained 32 children, 16 in each subsection, B I and B II. The eight cases of xerophthalmia occurred in the same subsection, B I, so that half the children in this subsection of the hospital were attacked. They fell ill almost simultaneously during May and the beginning of June. In none of the other subsections was the disease observed and no further cases have appeared in subsection B I.

There was no initial difference between the children of the two subsections, B I and B II; their ages and conditions of health were very much alike. The hygienic conditions were identical and everywhere excellent. The diet, however, was different inasmuch as the infants and the delicate children of Section A had chiefly whole milk, and puddings made of it. The older children in Section B had received a more varied diet; oatmeal gruel with moks or "ollebröd" (beer-and-bread soup) with a little fresh milk for breakfast. The midday meal consisted of milk pudding, sometimes made with buttermilk, oatmeal gruel, soups made with fruit juice and sometimes meat broth with pearl barley. As second course they had boiled fish, minced meat and a good helping of mashed potatoes. In the afternoon the children had cocoa with bread and margarine and in the evening milk pudding and bread and margarine. The margarine was made from vegetable fats and all the food was prepared with this vegetable margarine. The children were never given butter or eggs nor was cream or fresh milk ever used in the preparation of their food. The milk used consisted of half-skimmed milk. The only whole milk the children had was the small quantity given in the morning with the bread-and-beer soup.

The breakfast was somewhat different in the two B subsections. The matron of B II had given the children under her care beer-and-bread soup with whole milk, while the children of B I, where the xerophthalmia appeared, had had oatmeal gruel and rusks, so that these latter children had not had



whole milk for several months. The choice between these diets was left to the matrons' discretion, the rule being that children with a tendency to diarrhoea must not have bread-and-beer soup.

All the children were weighed regularly, the infants (Section A) once a week, the older children (Section B) every fortnight. The weighing always took place at the same time of the day, just before their last meal. As the weighing was done in the afternoon the results are not quite reliable. The amount of food the children had consumed, their activity and the quantity of sweat lost during the day must have affected the result. Another disturbing factor is that the weighing was rather infrequent. On the other hand it was always controlled by the same trained nurse and done at the same time and with the same scales. Care was also taken that as far as possible the children had emptied the bladder and rectum, so that the results undoubtedly gave a rough idea of the growth of the children.

The results obtained indicate that most of the children of Section A, who got whole milk, developed normally during the winter 1916-17 and the spring of 1917. Amongst the 32 children of Section B there were quite a number who only slowly put on weight during the winter; 15 kept their weight unchanged during winter and spring, while some of them even lost in weight, particularly during April and May. Of the 15 children five belonged to subsection B II and ten to B I. The eight cases of xerophthalmia occurred amongst the latter ten. The ages of these children varied from  $1\frac{1}{2}$  to 4 years. The cases are illustrated by the curves on p. 290.

The xerosis was very marked in all eight cases and had generally been present for some time probably for a couple of weeks before it was recognised. The first symptom was photophobia, the eyes became red and in some cases there was a slight sticky secretion. At first it was taken for a mild conjunctivitis, and treated with zinc drops. As this did no good and the eyes became worse, a more careful examination was made which made it clear that the children were suffering from xerosis. In some of the cases the xerosis was quite recent without general injection of the conjunctivae and in only two cases was there slight dryness of the corneae.

Apart from two cases one would undoubtedly, after a superficial examination, have considered these children quite normal. A more careful investigation, however, would have shown that they had been rather weak for some time and had lost their appetite. Their curves showed that six cases had remained constant in weight during the whole of the winter and that four cases had lost weight considerably during April and May, some time before the eye complaint was discovered (see Charts 1, 2 and 3).

Malling-Hansen's well-known investigations on the weight of children at the Deaf and Dumb Institute have shown that normal children during the ages of 9-15 years generally remain constant in weight or decrease slightly during the months of April, May and June. The loss of weight found in my cases during the spring has probably, to some extent, the same cause as that

noticed by Malling-Hansen. It is quite a common phenomenon that the change from indoor life to a life in the open air for most of the day is accompanied by a loss in weight which is soon compensated during the summer.

However, the loss of weight in my series of cases was greater than

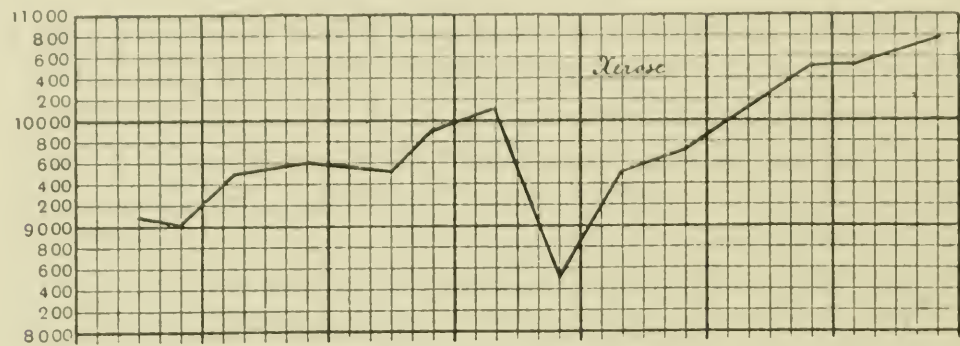


CHART 1. Weight record of *Allis*, born 2. vi. 1915. Admitted 24. v. 1917. Has been very ill with measles during the summer 1916. Since then she has developed fairly well but has lately again lost weight.

On 23. v. 1917. Xerosis conjunctivae of both eyes beginning. Cod liver oil—xerosis disappears and the child increases in weight.

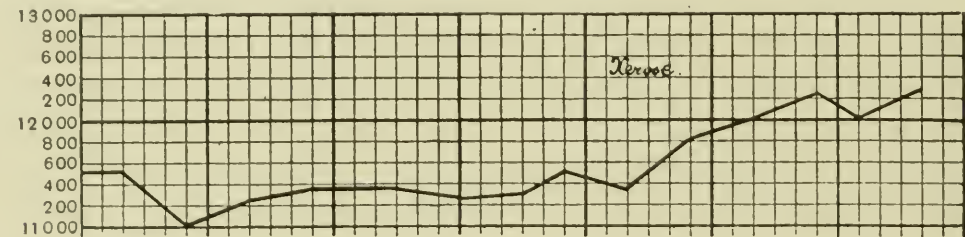


CHART 2. Weight record of *Sigurd*, born 19. x. 1915. Admitted 13. xii. 1915. Developed fairly well up to the winter 1916. Since then no increase but frequent attacks of eatarrh.

On 23. v. 1917. Slight xerosis of conjunctivae of both eyes. Cod liver oil—xerosis disappears and the child increases in weight.

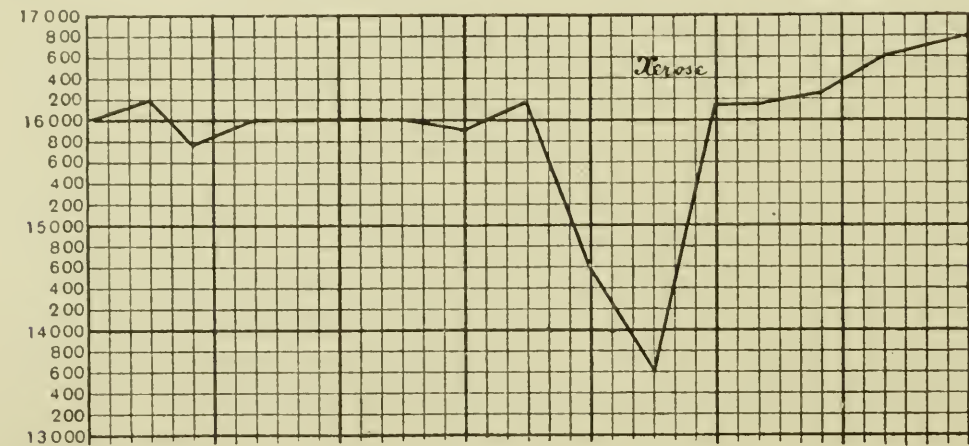


CHART 3. Weight record of *Hulda*, born 13. ii. 1913. Admitted 1. ix. 1913. She developed normally up to the winter 1916. Since then her weight has remained stationary.

On 23. v. 1917. For about a week she has been suffering from photophobia, has had purulent eyes and been treated for this with solutions of zinc. At present marked xerosis of conjunctivae of both eyes. Cod liver oil—xerosis disappears and the child increases in weight.



ordinarily takes place. It was also abnormal that the children's weight should have remained constant during the whole of the winter and spring when they were kept on a good diet and allowed to eat as much as they wanted. The loss or constancy in weight can only be explained as an inhibition of growth.

In spite of the loss of weight and the xerosis, it was decided to keep the children on exactly the same diet as before. Only the two children whose eyes were in the worst condition, were kept in bed for a few days. The others remained up and out of doors as before. The only treatment employed, in fact the only change in their mode of life, consisted in giving 10 gms. of cod liver oil twice a day. The children liked the oil and the effect was even more marked than in any of my earlier cases where the disease had progressed much further. In the course of a few days the xerosis became less and disappeared completely in a week. At the same time the children became livelier and those of them who had remained constant in weight for some time, began growing again. The curves clearly show the effect of the daily dose of 20 gms. of cod liver oil. In July the whole diet of the children was changed; but up to that time in spite of the same food as before, no more cases of xerophthalmia occurred.

It will be seen that the children behave much as the animals in the experiments of the American physiologists. They first stop growing and then lose weight. This is the initial stage which I have termed dystrophy. Then comes the eye trouble. The latter is therefore only a sign of a general pathological condition.

The reason for this is clear. The children who had plenty of fresh milk developed normally but amongst those who had only a trifling amount were several who did not thrive at all and some who lost weight, and many of them got bad eyes. Instead of whole milk these children had had "half-skimmed milk," *i.e.* centrifuged milk with the addition of enough of whole milk to make the fat content of the mixture 0.75 per cent. Both the whole milk and the "half-skimmed" milk had been pasteurised at the dairy and in addition both were boiled at the hospital before being given to the children.

As far as our knowledge went at that time the food given to the children in the Home was good and nutritious. The diet was sufficiently varied and contained plenty of protein, fat, carbohydrate and salt. It must also have been rich in water-soluble vitamins, on account of the liberal supply of potatoes and fruit juices. We now know that a mistake was made in not supplying the proper fats, *i.e.* fats in which the specific bodies are contained. As soon as these substances were supplied in the form of cod liver oil, the diet became a suitable one and the children thrived and the eye trouble disappeared. Our experience thus shows that vegetable margarine is unable to replace butter, and that it is important the children should be given whole milk.

Since July, 1917, the skimmed milk has been partly replaced by whole milk in the Children's Home. The children are now thriving better and that

their condition of health has improved. No further cases of xerophthalmia have occurred.

Of nine similar cases from the State Hospital, of ages varying from 9 months to 10 years, all showed xerosis conjunctivae. In four of the cases the cornea had not yet been attacked and with respect to the eye the disease was in its initial stage. The children had only recently had bad eyes, red and discharging, and developed photophobia. In three of these four cases night blindness could be demonstrated, a symptom which appears very early in the disease but was not noticed in any of my earlier cases, either because the children were too small, or because the lesion of the cornea was so extensive as to mask it. These cases and those from the Children's Home described above show that xerosis of the conjunctiva may exist for a long time without spreading to the cornea. In this early stage the complaint resembles an ordinary conjunctivitis, the eyes often being red, discharging and showing photophobia. Much conjunctivitis, however, appears later and generally in children which are suffering from catarrh.

In the remaining five cases the cornea was attacked, in three cases with keratomalacia of one eye, two of which displayed complete necrosis of the cornea; in two cases with keratomalacia of both eyes. The children were very backward, weighed less and were much smaller than normal children. They were weak and anaemic and many of them seemed to behave in a peculiar manner to infections, particularly catarrhal infections of the mucous membranes and infections of the urinary tract. Six of the nine children had catarrhal infections, two of them, broncho-pneumonia, and three, pyuria. I intend to return to this point later. Not only were the children weak in body but their minds were also affected. In the early cases the children were quieter than usual and had lost their appetite. In the more advanced cases they were extremely sensitive to external stimuli. This may have been partly due to the eye complaint but, no doubt, was mainly caused by the malnutrition. In my previous articles I have termed this condition psychic hyperaesthesia. This extreme sensitiveness has nothing to do with the nervous symptoms met with in convulsions nor with the "hypermotility" described by Czerny as characteristic of incipient "Mehlnährschaden." I have found neither of these symptoms occurring in this kind of dystrophy (alipogenetica) or in carbohydrate dystrophy.

The above cases illustrate the condition described in my earlier paper as the third type of xerophthalmia. The first sign seems to be merely an inhibition of growth passing into dystrophy, of a type found amongst children who have been fed chiefly on separated milk for some time. Amongst these cases were three which had had whole milk for a long time and one had even been given cream, yet they all had xerophthalmia and the three had marked dystrophy just like the children fed mainly on centrifuged milk. These cases do not



agree altogether with my previous experience. The explanation must evidently be sought in the animal experiments of the American physiologist who found that the specific bodies dissolved in milk fat are easily destroyed. In the three cases under consideration the whole milk was first mixed either with centrifuged milk or with water or oatmeal gruel and then boiled for a considerable time. The cream given in one case was the so-called "export cream," specially prepared and sterilised in order to preserve it. These cases were cured in exactly the same way as all the others by administering the specific bodies in the form of cod liver oil and whole milk.

All the above cases lead to the conclusion that xerophthalmia, inhibition of growth and dystrophy, are due not to the absence of water-soluble vitamins or of fats but to the absence of the specific bodies present in certain fats.

#### Type II. *Xerophthalmia with Carbohydrate dystrophy.*

Among the 23 cases of xerophthalmia treated in 1917 were six cases displaying the same clinical picture as those described by Czerny-Keller under the name of "Mehlnährschaden." Nearly all of them belong to the form, which, in an earlier paper, I described under the name of carbohydrate dystrophy. The diet of these children had for a long time been very uniform and consisted mainly of carbohydrates. All the children were infants, only one was more than 12 months old. Three of them were less than six months. All the children were backward in development. They had lost their appetites, and were fretful and sensitive to outside impressions when they had not been rendered apathetic by the ravages of the disease. They were nearly always anaemic and most of them frequently suffered from catarrh. Two had bronchopneumonia and four had pyuria. The eye complaint was far advanced in all the cases. The corneae were always attacked, in one case with complete necrosis of one cornea and in another (which died) with complete necrosis of both corneae.

All these cases showed the symptoms characteristic of carbohydrate disease. This was not so with the cases previously described. The tissues were oedematous and the children had the appearance of being blown out. There was also muscular rigidity which was so pronounced in three of the cases as to constitute the characteristic muscular hypertonicity (see Pl. II, figs. 1 and 2). The usual treatment with cod liver oil and with whole milk or human milk caused all symptoms to disappear.

The question now is, what is the aetiology of the oedema and hypertonicity of the muscles? These symptoms cannot be ascribed to the absence of the specific lipid bodies as they did not occur in the other cases where the disease was due to this cause. It might perhaps be suspected that the oedema was caused by the inability of the patients to excrete the necessary quantities of salts. This assumption, however, is incorrect, as all the patients were able to excrete salts to the normal extent. It is more reasonable to associate the oedema and the hypertonicity with the excessive supply of carbohydrates

and the simultaneous almost complete absence of fats, the points in which these cases differ markedly from the others. It must not be forgotten, however, that the oedema and hypertonicity may be due to the absence of quite different vitamins or of other specific bodies. In that case the whole clinical picture included in "Mehlnährschaden" would come under Casimir Funk's so-called "Avitaminoses" if the particular lipid bodies were classed as vitamins.

The case quoted below may possibly throw some light on this question.

Ernst, 4 years old. Son of a bricklayer. Admitted to hospital 14. ii. 1914; discharged 29. iv. 1914.

During the last two years the patient's evacuations have had a tendency to be frequent, thin and slimy. He has repeatedly been on constipating diet for a considerable period but without appreciable effect. There has never been blood in the evacuations and the patient has never suffered from constipation. He has always been very keen on eating potatoes and has also had an inclination to eat gravel, soil and paper. The mother thinks he has a longing for these things, he has been known for example to peel the plaster off the walls and eat it.

During the last six weeks his condition has become worse. He has had as many as 10-20 thin, slimy and greenish evacuations daily. Both faeces and urine are often passed involuntarily. In the last few days oedema of the eyelids and legs has appeared. All this time he has been kept in bed on a diet consisting of oatmeal gruel, bread and mashed potatoes. He has eaten a great deal and during most of his illness has been on a milk-free diet.

On admission he was very wasted. There was cyanosis of the hands, and the mucous membranes were pale. There was extensive general oedema of the subcutaneous tissue especially of the face. The eyelids were so oedematous that the eyes were almost closed. There was also considerable oedema of the extremities and loins. There was a considerable amount of ascites. The urine was normal.

The patient was treated with tea, oatmeal gruel, boiled milk, acorn cocoa, and milk pudding. The motions, which previously were slimy, thin and green, and had a sour odour, improved and the bowels acted normally a fortnight after entering hospital. Simultaneously the oedema disappeared giving rise to a loss of about 1 kg. in weight. After this the patient speedily recovered and he put on 2.5 kg. in weight.

In this case the oedema had appeared after prolonged feeding with carbohydrates and potatoes. Perhaps this combination of foodstuffs accounted for the unusually marked and widespread character of the oedema. I have never before seen it so extensive after a carbohydrate diet. The child must have been supplied with large quantities of water-soluble vitamins in the potatoes so that the oedema cannot be ascribed to the absence of these bodies. Since a lack of the specific lipid bodies is not known to cause oedema it is probable that the absence of the fats in addition to the liberal supply of



carbohydrates, may have been the cause. At all events it disappeared in this case when the amount of carbohydrate was reduced and replaced by fats in the form of boiled whole milk.

Complaints similar to the above have so far been little known in adults. During the war, however, there have been epidemics of dropsy, the so-called war-oedema, occurring in places where famine was reigning and in the large prisoners' camps in Germany. As far as is known this disease is associated with similar symptoms and arises in connexion with the same conditions of diet. Concerning the aetiology of this war-oedema nothing so far is known beyond the fact that it is connected with a deficient diet, but probably similar causes to those in the case of children are operative.

#### *Notes on Treatment.*

The treatment of all the cases has been identical with that described in my earlier papers.

The local treatment of the eyes has generally been confined to frequent washing with sterile water, when there was marked conjunctivitis. In cases of xerosis, sterile vaseline was applied. When the cornea was attacked and there was danger of necrosis or when ulceration had already appeared, atropin drops were used. In two cases it was necessary, on account of panophthalmia, to remove one of the eyes.

The chief aim has always been to improve the children's general condition of health by supplying them with the necessary bodies which had been absent from their food. As before, cod liver oil was found to be a specific cure for the disease. Its effect was always very marked, but most striking in the early stages of the complaint. The eyes improved within a few days and became quite normal after about a week's treatment. The loss of weight was simultaneously arrested and the children generally began to thrive well in a short time. That this improvement was due to the cod liver oil alone is shown by the result of treatment of the eight cases from the Children's Home in Copenhagen, described on p. 288. Amongst my earlier material there were two cases which showed that cod liver oil with ordinary boiled milk and milk pudding had a better and speedier effect than unboiled whole milk given alone. The same seems to have been true of a case where the child had had unboiled fresh milk and orange juice for some time. Though the general condition was considerably improved by this treatment, the eye complaint was only cured after the administration of cod liver oil.

The 15 cases treated at the State Hospital were given a diet corresponding to the children's age and development, designed to supply them with as much fresh milk as they could digest. The milk was always boiled. Three children under 6 months old had human milk at first. The boiled milk was good fresh milk heated to boiling point and then quickly cooled. The experiments on animals carried out in America have shown that the specific bodies will stand a short boiling but that they are destroyed by prolonged heating. The

same apparently is true for cod liver oil. The product used was always the ordinary Norwegian “steamed medicinal cod liver oil.” This has only been heated to 50° C. It is possible that other preparations which have undergone a more drastic treatment will be found to have no specific effect at all, as by such a handling the lipoid bodies may have been rendered inactive.

The result of the treatment of the 23 cases here described was that only one died. The remainder recovered. The child that died had total necrosis of both corneae. The cause of death, which took place 11 days after entering hospital, was an attack of broncho-pneumonia. Only one of the 22 children which recovered became completely blind. On admission this child had complete necrosis of both corneae. Normal sight of both eyes was restored in the 13 cases which were taken in time. Normal sight of one eye was restored in five cases and three children were discharged with indifferent sight of both eyes. The results obtained were thus considerably better than in the earlier cases. This was due to the fact that the disease was generally recognised earlier. The treatment has proved so reliable that it is always possible to save the sight provided the treatment is started before the corneae have become necrotic.

Of the 15 children treated at the State Hospital during 1917 only one (a three months’ old child with carbohydrate dystrophy) came from Copenhagen, the other children were from the country. The table below shows that the number of cases from the country and their distribution was very much as in 1916.

The children from the country nearly all came from the poorest and most destitute homes, those of labourers, herdsmen, outdoor servants and the poorest cottagers, and they nearly all bore evidence of extreme poverty and neglect. Almost all came from the country proper, and only a few from provincial towns. Most of them came from Zealand, but there were also cases from the other islands and from Jutland.

The main reason for the malnutrition of the children must undoubtedly in most cases be sought in the parents’ want of sufficient means of subsistence. The high prices forced them to give the children the cheapest possible food,

Table I.

		Age of the patients								
		From the country	From Copenhagen	From the country			From Copenhagen			
				2-6 months	6-12 months	= > 1 year	2-6 months	6-12 months	= > 1 year	
1912	4	2	2	—	1	1	1	1	—	
1913	9	8	1	4	3	1	1	—	—	
1914	8	6	2	2	1	3	2	—	—	
1915	11	8	3	1	7	—	1	2	—	
1916	17	14	3	1	5	8	2	—	1	
1917	23	14	9	2	7	5	1	—	8	
Total	...	72	52	20	10	24	18	8	3	9



and the cheapest is, as is well known, the poorest in fat. One would have thought it would have been comparatively easy to get unskimmed milk in the country. However, just the opposite seems to be the case. The parents always said that it was difficult to get unskimmed milk unless one had a cow oneself. All the unskimmed milk went to the dairies. It was easy, however, to get both centrifuged milk and buttermilk, both of which remained cheap, while the price of unskimmed milk kept on increasing.

The reason for the somewhat larger number of town cases in 1917 is entirely accounted for by the eight children from the Copenhagen Children's Home. It is significant that these cases occurred because the children in this Home during 1917 had been on a diet similar to that supplied in the poorest cottagers' homes, namely pasteurised skimmed, instead of whole, milk.

There are two other conditions besides diet which are of considerable importance for the development of xerophthalmia. These two factors are: *the relation of xerophthalmia to the time of year* and *the effect of other infections on the appearance and course of the disease*.

With regard to the influence of the seasons the accompanying Chart 4 shows that most of the cases appeared during the three months, March, April and May. The largest number occurred in May. Of the 23 cases during 1917, 18 occurred in this three-month period.

Of the 1917 cases there were eight in which, in addition to the eye complaint, the only abnormality was inhibition of growth. If there had been no opportunity of following these cases from the Children's Home over an extended period, so that the loss of weight and inhibition of growth could be observed, little beyond the eye trouble would have been discovered. In these uncomplicated cases the eye complaint appeared about the same time in May. This result can hardly be accidental; it has been true of the incidence of xerophthalmia year after year (Chart 4), and there must undoubtedly be some natural cause. In my earlier communication I pointed out that it could hardly be due to the diet. The children had had almost the same food both winter and spring. I then put forward the suggestion that it might be connected with the children's growth period.

Malling-Hansen, by his studies at the Deaf and Dumb Institute in Copenhagen, has shown that there exist quite definite periods of growth in children, and his results have been confirmed by Camerer and Schmid-Monnard. From these investigations it would appear that during the year there are three periods of growth as measured by increase in height, viz. a minimum, an intermediate, and a maximum period. The minimum period starts in August and lasts until the end of November. During this time children hardly grow at all. In the intermediate period from the end of November to the end of March, the children grow twice as much as during the minimum period. From the end of March up to well into August the growth amounts to two-and-a-half times that of the minimum period. The growth period of children is

therefore preeminently from November till August and is greatest in the months of spring.

In Chart 4 the occurrence of xerophthalmia in relation to the seasons is shown in company with Malling-Hansen's curve of the three periods of growth, and it will be seen how accurately they agree, the greatest number of cases of xerophthalmia coinciding with the maximum period of growth. If the cases of infants with carbohydrate dystrophy are excluded, all cases of xerophthalmia will be found to have occurred during the two periods of

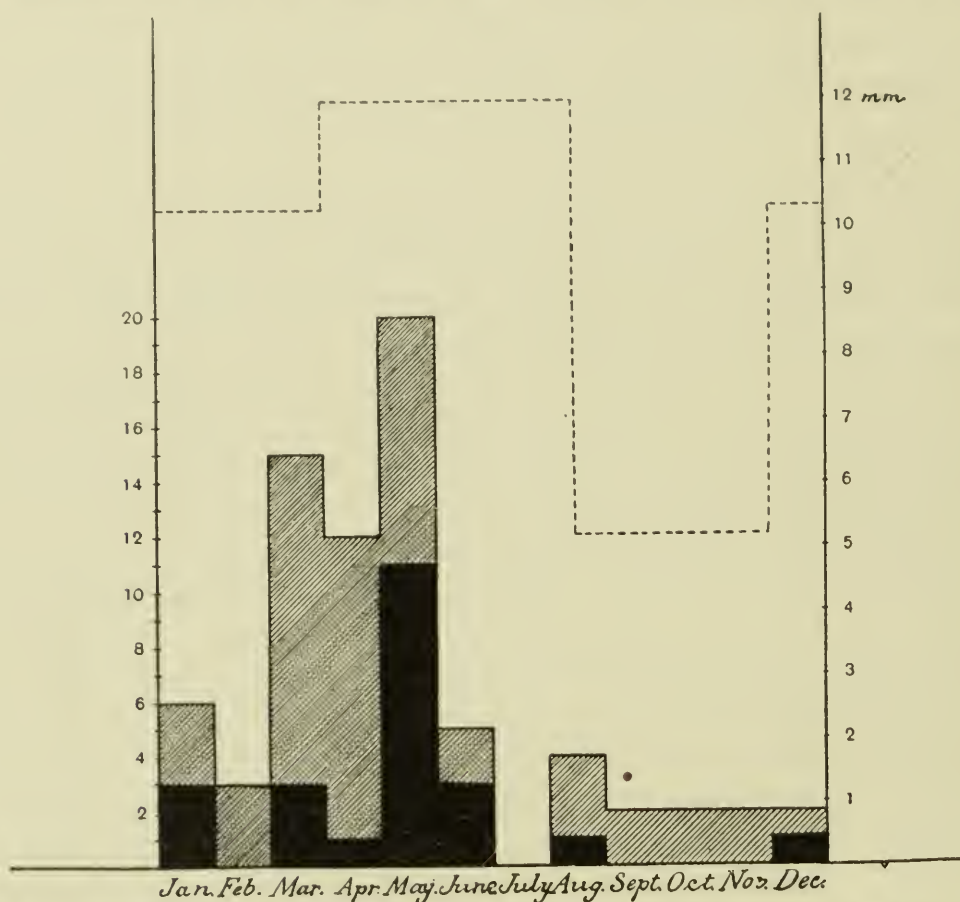


CHART 4. The broken line gives the seasonal periods of growth according to Malling-Hansen. The shaded columns record the seasonal incidence of xerophthalmia (admission to hospital) for 63 cases, 1911-1917.

The black columns give a similar record for 23 cases from 1917 onwards.

growth and none during the minimum period. Of the uncomplicated cases all are found to have occurred in the second month of the period of maximum growth.

These facts can be best explained by assuming that the specific lipid bodies essential for growth are being continually used up while growth is taking place. One could easily imagine that the specific substance took part in the formation of new tissue or, what seems more probable, that the lipid bodies were necessary for the glands on whose proper functioning growth depends. Funk holds this view with regard to the influence of vitamins on growth.



As xerophthalmia is caused by the absence of bodies essential for growth, it is clear that the disease will predominate during that part of the year when the organism consumes the largest quantities of lipid bodies, for its growth, *i.e.* during the maximum period for growth.

As already pointed out, the majority of my cases support this contention, particularly the uncomplicated ones. Some cases, however, appeared earlier, *i.e.* during the intermediate period of growth. In these cases there must be other causes which are *also* operative. This leads us to the other important aetiological factor: the *influence of other infections* on the appearance and course of xerophthalmia.

From a study of my cases it is evident that whenever xerophthalmia breaks out before the maximum period of growth, acute and chronic infections have always been present for some time, while in uncomplicated cases xerophthalmia occurs within the maximum period. The only exceptions are the rare cases of carbohydrate dystrophy in infants, but here the conditions of growth are probably quite different (Friedenthal). We see therefore that only those cases with a chronic infection make their appearance in advance of the usual time. This indicates that the infection contributes or predisposes to xerophthalmia.

Xerophthalmia may be cured by supplying the specific lipid bodies. The same is the case with regard to the inhibition of growth. The children begin to thrive again when given cod liver oil. The cases following in the wake of other infections behave similarly. Clinical experience has shown that the only way of curing all these infections is by strengthening the children's general constitution by supplying the absent specific bodies in the form of cod liver oil, whole milk, eggs, etc. At the State Hospital no other treatment has been used and the infections were nearly always completely cured when the children were discharged.

The grave prognosis which previously obtained in cases of xerophthalmia was linked with this question of secondary infections. So serious indeed was the outlook that children, as was the case with young rats, died if a change to a proper diet was not made. The reason why young rats died is not explained but in the case of children the infections are the cause. It is absolutely characteristic of these dystrophic children, how little able they are to withstand infections and how quickly they die of a serious intercurrent infection.

It will be seen therefore that there are three aetiological factors responsible for the origin and the development of xerophthalmia:

Firstly, the *diet*, which does not contain, or at any rate is *poor in the specific lipid body or bodies*.

Secondly, *growth*; xerophthalmia is found particularly in children, or animals who are growing.

Thirdly, *antecedent infections of long duration*. The explanation of this is uncertain, since no animal experiments have yet been brought to bear on the subject. It is, however, reasonable to suppose that the specific lipid bodies

are necessary for formation of antibodies against infections and that they are continuously used up in the process as in the case of growth. Antibodies may indeed be looked upon as a kind of internal secretion. Thus it will be easily understood that patients suffering from "Mehlnährschaden" and from the special form of dystrophy here elucidated, are extremely susceptible to all infections and have very little capacity for resistance.

This would explain why xerophthalmia in earlier days was discovered usually in the final stages of prolonged disease such as tuberculosis and typhoid fever. We also get an explanation of the well-known ancient clinical observation, which is certainly true, that foods such as whole milk, cream, egg, and cod liver oil are particularly important for children when they are growing most, just as they have been shown to be the best foods in the convalescent stages after infections and in the treatment of chronic tuberculosis.

#### SUMMARY.

1. There are fats which are indispensable for children because they contain specific bodies, essential for normal growth.

2. If these lipid bodies, termed "fat soluble A bodies" by McCollum, are absent from the food for a long time, an inhibition of growth will occur and the conditions which I have termed *Dystrophia alipogenetica* will finally appear. This condition involves a great susceptibility and lowered resistance to all infections and often leads to xerosis of the conjunctivae and corneae associated with night blindness. The xerosis has a great tendency to result in keratomalacia. In its first stage xerophthalmia resembles a slight conjunctivitis; the children develop photophobia, their eyes are red and there is a slight secretion.

3. Xerophthalmia generally occurs in spring, the time when growth is at its maximum.

4. The disease is most frequent amongst the children of the poorest country folk and is always due to an unsuitable artificial diet, generally the replacing of whole milk partly or completely by centrifuged milk, butter milk or concoctions of flour. It may appear in children who have received whole milk and cream, but in that case the milk and cream have been boiled for too long a time or have been subjected to other kinds of drastic treatment which have destroyed the specific lipid bodies.

5. Xerophthalmia is easily cured when recognised in time. The best treatment is cod liver oil, but whole milk and probably cream, butter, eggs and other fats containing the specific lipid bodies are also curative. It is important to remember that these foods should only be subjected to the ordinary short boiling.

6. The disease is fairly common in Denmark, apparently more so than in other countries. It is, for instance, practically unknown in Sweden and Norway. According to Mori's statistics it used to be rare in Germany. The



reason for its being so frequent in Denmark is probably partly due to the fact that this country exports most of its dairy produce, especially butter.

7. Many of the cases of blindness and leucoma attributed to eye complaints during infancy, are perhaps due to xerophthalmia. Of late it has been said that blindness amongst children in Denmark is increasing while gonorrhoeal ophthalmia in new born infants at the maternity hospital is decreasing. Ophthalmo-blenorrhoea has been the cause of blindness in very few of the recent admissions to the blind institute, all of which supports my contention that xerophthalmia is the actual cause of the large number of blind children in Denmark.

## REFERENCES.

- BLOCH, C. E. (1918). *Rigshospitalets Borneafdeling Meddelelser*. II.  
 CZERNY, A. and KELLER, A. (1906). *Des Kindes Ernährung* etc.; Leipzig.  
 FRIEDENTHAL, H. (1913). Über Wachstum. *Ergebnisse d. inn. Med. und Kinderheilk.* XI.  
 FUNK, C. (1914). *Die Vitamine*, etc., Wiesbaden.  
 JENSEN, E. (1903). Xerophthalmia amongst children. *Hospitalstidends*.  
 LANGSTEIN and EDELSTEIN (1917). *Zeitschr. f. Kinderheilk.* XVI.  
 MCCOLLUM and DAVIS (1915). *Journ. Biol. Chem.* XX, XXI, XXIII.  
 MCCOLLUM and SIMMONDS (1916). *Ibid.* XXIV, XXV.  
 ——— ——— (1917). *Ibid.* XXXII.  
 MALLING-HANSEN (1886). *Periods in the growth of children and in the sun's heat*, Copenhagen.  
 MORI, M. (1904). Über der sogenannten "Hikan," etc. *Jahrb. d. Kinderheilk.* LIX.  
 OSBORNE and MENDEL (1914). *Journ. Biol. Chem.*  
 PITZ and KENNEDY (1916). *Ibid.* XXIV, XXV.  
 RÖNNE, R. (1916). Über die Aetiologie und Behandlung der Keratomalacia. *Klinische Monatschrift f. Augenheilk.*  
 STEPP, W. (1917). *Ergebnisse d. inn. Med. u. Kinderheilk.* xv.  
 TSHIHARA, S. (1913). Zur Aetiologie der idiopath. Hemeralopie bzw. Xerosis conjunct. *Klin. Monatschr. f. Augenheilk.*

## APPENDIX.

OBSERVATIONS ON XEROPHTHALMIA IN DENMARK DURING  
1918 AND 1919.

From Table I above and Chart 5 it will be seen that there is a sharp rise in the number of cases from year to year right up till 1918. It is particularly during the hard times consequent upon the war, in 1915, 1916, and 1917, that the cases are so frequent. If we now consider the districts the children came from, we find there are 52 from the country and only 12 cases from Copenhagen if we except the eight cases from the Children's Home. All the most serious cases came from the country, having arisen in connexion with a diet composed principally of centrifuged milk, bread, potatoes, and margarine, and it is these cases which have increased so markedly during the war.

That there have been more patients from the country is due to the fact that all through the war care has been taken that there was a comparatively

plentiful supply of fresh milk in Copenhagen for children and that the cost was reasonable even for poor people. In the country the conditions were difficult as already mentioned.

During 1918 a change suddenly occurred, the number of cases treated by me falling from 23 in 1917 to 1 in 1918.

The single case which was admitted to the State Hospital in 1918 had only an exceedingly slight degree of xerosis of the conjunctivae. It was an emaciated infant with marked carbohydrate atrophy, arising from a diet of barley water during a long period.

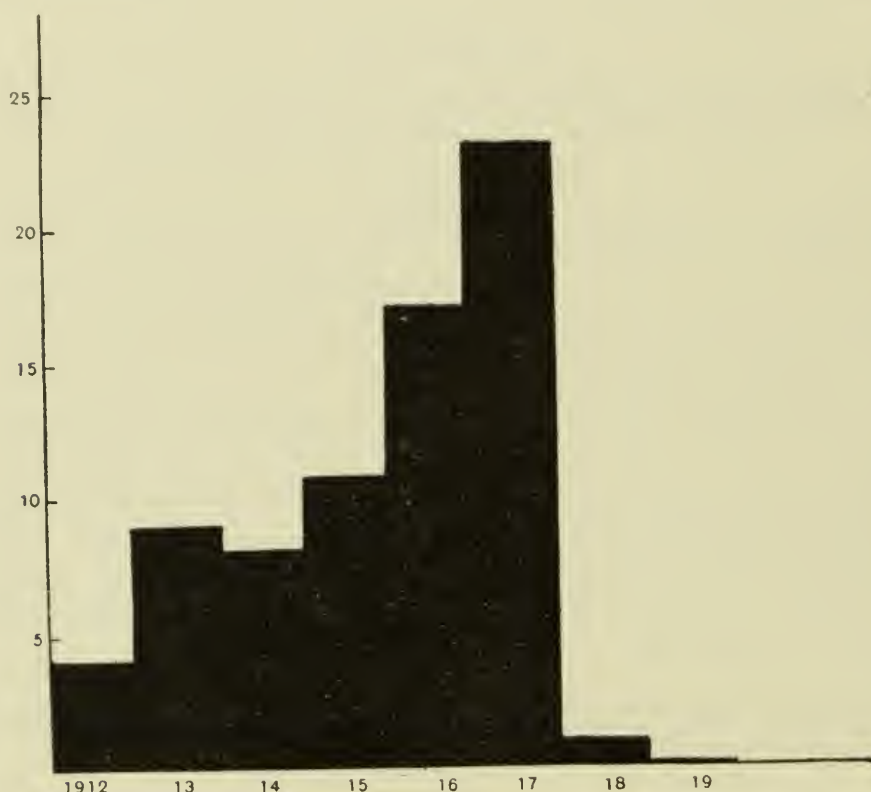


CHART 5. Recording the number of cases of xerophthalmia admitted to the State Hospital, Copenhagen, 1912-1919.

There have been no cases treated in the State Hospital in 1919 (up to July 1st); from the experience of previous years it is not to be expected that any cases will arise now that the season of the year is past when the disease usually appears.

It is impossible that this decrease can be due to the fact that cases in 1918 and 1919 were being treated at other hospitals than the State Hospital. This is the only civil hospital which is owned and managed by the Danish Government and it therefore receives patients from the whole country, from towns as well as country districts, and deals preferably with difficult and serious cases. Thus the diseases which are treated in the course of the year at the State Hospital are always a good sample of the serious affections which are spread throughout the whole country (acute infectious diseases excepted).

The sudden decrease in the number of cases shows therefore, that after



being a rather frequent and serious condition, the disease had suddenly become uncommon or mild.

The Ophthalmological Society of Copenhagen has this year (1919) undertaken a statistical investigation of the incidence and distribution of xerophthalmia in Denmark, through Dr O. Blegad. The work is not yet finished, but it already appears that about 400 cases have been discovered from 1908 to 1917 (inclusive), and that the number has risen considerably during the war. For the year 1918 only a very few cases (six to eight) could be collected and none at all for 1919. This corroborates my contention that xerophthalmia was formerly a rather common disease in Denmark, that it increased greatly during the war and practically ended in 1917.

The reason of this cannot be that the period of scarcity became less acute during 1918 and 1919. The opposite is the case, as is well known. When the German submarine blockade became established in February, 1917, the import of fodder for cattle was completely stopped. The result was that the production of milk was more reduced than before and prices for fresh milk, cream and butter reached hitherto unknown heights.

It is possible that the decline of the disease in 1918 may to a small extent be accounted for by the appearance of my articles on the subject in 1916, 1917, and 1918, and the discussion to which they gave rise. Every Danish doctor had his attention drawn to this disease. This may have played a part especially with regard to those cases which arose from an excessive diet of carbohydrates, in that the doctors may have changed the food in time. It cannot however apply to the large number of severe cases from the poorest homes,—where the children had lived on centrifuged milk, margarine, lard, bread, potatoes and the like, because in these cases marasmus is often the only manifestation of the disease until the point when the eyes are attacked.

The only possible explanation seems to be the radical change of food which took place after December, 1917, for the whole population, particularly, however, for the poor. From February, 1917, the German U-boat blockade put an end to the importation of fodder for cattle, and of all fat-stuffs, including raw products for the production of margarine. Consequently pig-rearing became limited and the manufacture of margarine ceased completely in the course of a short time. As previously pointed out, lard, meat and above all margarine are the fatty foods on which the greater portion of the Danish population live. When the supply of margarine and lard failed in 1917 and no more was forthcoming, butter was the only fat left. The blockade was also responsible for a largely decreased output of butter and prices became impossible for the masses of the people. Thus it became necessary for the State to take over control of all butter. From December 21, 1917, butter was rationed so that everybody, adult and child, was entitled to receive 250 gms. a week, and it was sold at such a low price that all could buy it.

*From that moment everyone ate butter instead of margarine and since then there has been no xerophthalmia in Denmark.*

It will be seen how this great dietetic experiment involving a whole nation, accurately agrees with the American and English animal experiments and with my earlier clinical observations.

On the other hand it is impossible with certainty to throw any light on the extent to which change of diet in 1918 and 1919 affected the two other conditions associated with xerophthalmia in young children, viz.: dystrophy and the reduced power of resistance towards infections. In addition to change of diet there are so many other circumstances which must have had an influence on the morbidity and mortality of these two years. So much however can be said that the morbidity and mortality in 1918 and 1919 have been comparatively slight taking into account the great influenza epidemic. Now that the war is over, the manufacture of margarine will be started again and the rationing of butter will cease, so that, on account of its cheapness, margarine will supersede butter as before. It is therefore to be feared that next spring this disease of malnutrition and the disastrous ophthalmic condition will occur again amongst the children of the poorer people in Denmark.

## EXPLANATION OF PLATES II—IV.

### PLATE II.

Fig. 1. Atrophy with keratomalacia, dry scaly skin, stiff neck, arms in the "paw position," infant extremely impoverished and wretched. Type 1.

Fig. 2. *Anna, M. J.* Three months old. Carbohydrate dystrophy with oedema. Type 2.

Fig. 3. *Jörgen, J.* Two years old. Dystrophy and xerophthalmia with total necrosis of both corneae. The disease arose from feeding with centrifuged milk. Type 3.

### PLATE III.

Fig. 4. *Gunner Willy N.* After recovery. The child can see well; right eye almost normal with a little spot situated upwards and inwards; left eye partly staphylomatous.

Double-sided keratomalacia with complete necrosis of left cornea; right cornea generally hazy, with ulceration on its upper and inner aspect.

The disease arose in a 16 months' infant after feeding from the age of six months with centrifuged milk, milk pudding, oatmeal porridge, etc.

Treated with cod liver oil, fresh milk, and milk pudding.

Fig. 5. *Leo A.* After recovery. The child can see a little with the right eye which is partly leucomatous. The left eye is wasted.

Double-sided keratomalacia, total necrosis of left cornea with perforation and expulsion of lens, etc.; half of the right cornea necrotic, also with perforation.

The disease appeared in a 14 months' infant after feeding with centrifuged milk, rusks, etc. (The child never received any fresh milk.)

Treated with cod liver oil, fresh milk, and milk pudding.

### PLATE IV.

Fig. 6. *Yrsa N.* After recovery. The child can see with the right eye which is almost normal, the left eye being for the most part leucomatous.

Double-sided keratomalacia with total necrosis of the left cornea and less necrosis of the right, in an infant three months old.

The disease arose after about two months' feeding with oatmeal.

Treated with cod liver oil, human milk, and milk with other things.

Fig. 7. *Vilfred H.* After recovery. The child is blind, left eye wasted, right eye staphylomatous.

Double keratomalacia, with widespread necrosis of corneae, in a two months' old child.

The disease arose after two months' feeding with oatmeal gruel.

Treated with human milk.





Fig. 1



Fig. 2



Fig. 3





304<sup>2</sup>



Fig. 4



Fig. 5





# THE WATER SUPPLY OF THE EGYPTIAN EXPEDITIONARY FORCE, WITH SPECIAL REFERENCE TO THE EFFICIENCY OF MECHANICAL RAPID FILTRATION WITH CHLORINATION.

BY R. J. S. McDOWALL, M.B.

*(From the Physiology Department, University of Edinburgh: late S.S.O. in charge of Water Supplies, Palestine L. of C.: and late D.A.D.M.S., E.E.F.)*

THE provision of an epidemiologically safe water supply to the Egyptian Expeditionary Force, as it fought its way across the Sinai Desert to deliver Palestine from the hand of the Turk, will long be remembered as one of the most remarkable achievements of the war.

The route was already historic, as along it the ancient kings of Egypt and Assyria had led their hosts against each other. Napoleon followed the same route in 1798, and Sir William Willcocks is of opinion that the Israelites also used it. The 17th chapter of Exodus gives us an insight into their difficulties regarding water supply.

For a modern army, however, the problem was more difficult, for even the army of Napoleon was in numbers less than one-tenth that of the Egyptian Expeditionary Force. Along the first part of the route is a chain of wells capable of supplying small numbers of troops, and this sufficed for the needs of the Australian and New Zealand cavalry which formed the advance guard, but it was quite inadequate for the main army. The eastern part of the Sinai Desert is practically waterless, and it was on the other side of this waterless area between Gaza and Beersheba that the Turks had entrenched themselves and actually managed to hold up our advance for over a year. It was therefore necessary to provide the army with water from a source outside Sinai, and the only one was the Fresh Water Canal which takes the water of the Nile to Port Said and the other stations on the Suez Canal.

When it is considered that this canal leaves the Nile near Cairo and flows through some 70 miles of the Delta, where it is exposed to every conceivable pollution, and that in addition to being a veritable cesspool it is also bilharzia-infected, it will be seen that the problem of purifying the water for consumption by troops was no easy one.

The primary question to be solved was whether the slow sand filter or the mechanical rapid filter should be used.

It is interesting to note that at home the Metropolitan Water Board, forced by the exigencies of war which demanded economy of coal and labour, had set

themselves a similar problem. In the thirteenth Research Report, Sir Alexander Houston sums up the question at issue thus:

“Is it permissible to filter stored water so rapidly as to create a material economic gain in the saving of filtration area, yet by the aid of anti-filtration or post-filtration sterilisation processes to produce a water which is epidemiologically safe, innocuous, tasteless and reasonably satisfactory from a physical and sentimental standpoint?”

Rapid filters have been extensively adopted in America. On the other hand British opinion, influenced by the possibly not altogether disinterested advice of engineers and supported by the results of certain chemical analyses, largely adheres to the slow filtration method which “has stood on the whole favourably the test of time.”

In Egypt, however, there was no doubt as to the gross contamination of the supply, and it was realised that it was of negligible importance what the results were as regards albuminoid nitrogen, ammonia or oxygen absorbed, so long as a water could be provided which was *bacteriologically safe*. Moreover at Kantara, which was the base of the advancing force, space had to be economised, as had money and labour, while the occurrence of sand-storms might, it was thought, seriously interfere with the working of slow sand filters. It was therefore decided to adopt the method of sedimentation, rapid filtration and chlorination.

Six similar plants were installed at different military posts of the Suez Canal Defence, and it is interesting to note that when the Turk was driven from the immediate vicinity of the Suez Canal, one of these plants was transferred from a smaller post to Kantara where it was subsequently connected with the historic pipe-line across the Desert. Such an economy would not have been possible had the slow filtration process been adopted.

The type of plant used was a modified “Jewel.” In the first instance, the water passed through a strainer of brass wire gauze with a mesh of a sixteenth of an inch to exclude the snails which are the intermediate hosts of worms causing bilharziasis. Thence it was led to a settling tank on the maze principle, on entering which a solution of aluminoferric was added. The water took some ten hours to flow through the tank, and by this treatment alone the transparency was increased four or five times and 40 per cent. to 60 per cent. of the organisms removed. It then passed to the filters.

Each filter consisted of an inner steel cylinder, containing 40 inches of coarse sand supported on gravel, and an outer cylinder which was three feet higher. The water rose between the cylinders at a speed which allowed of a maintenance of a head of  $2\frac{1}{2}$  feet above the sand level and after passing through the filter-bed of the internal cylinder, reached the exit pipe through perpendicular strainers of perforated brass. The filters were periodically cleaned by reversing the direction of the flow and raking the surface of the sand. After washing, it was necessary to run to waste for twenty minutes to allow the sand surface to settle. The running to waste occurred automatically, indeed all the



valves were so arranged that the process was quite fool-proof. On leaving the filters, the water was quite clear, and 95 per cent. of the total organisms found to have been removed.

The water was then conducted to storage tanks, at the entrance to which it was chlorinated to the extent of usually 0.75 to 1 part chlorine per million of water. The actual method of chlorination differed at some of the tanks, but, so long as efficient mixing was accomplished, it did not appear to matter which was used. The amount of bleaching powder to be added was gauged by a modified form of the ordinary Horrocks test, a modification of which was also used for the testing of the bleaching powder itself.

From the bacteriological point of view, the process since its installation has given the utmost satisfaction. Samples have been taken weekly and an average series of results is given below.

*Summary table of bacteriological results based on 3½ years' experience and the examination of multiple samples.*

Source of sample	Colony count per c.c.	Glucose fermentation	Lactose fermentation	"Flaginae" <i>B. coli</i>
Fresh Water Canal	1200	in 0.05 c.c.	in 0.05 c.c.	in 0.1 c.c. often in 0.05 c.c.
After sedimentation	800	not in 0.05 c.c.	not in 0.05 c.c.	not in 0.05 c.c.
After filtration	50	not in 0.5 c.c.	not in 0.5 c.c.	not in 1 c.c. often not in 5 c.c.
After chlorination	20–30°*	not in 10 c.c.	not in 10 c.c.	not in 10 c.c.

\* Developed chiefly, if not entirely, from non-pathogenic spores unaffected by the chlorination.

The freedom from bilharzial infection was presumed, as all water was stored snail free in the plant, reservoirs and pipes 36–48 hours before actual consumption.

It was originally laid down that no water which contained *B. coli* in 1 c.c. should be consumed by troops in the E.E.F. As, however, the examination took several days to carry out, for practical purposes a test with quantities varying from 0.5 c.c. to 10 c.c. of water for the presence or absence of acid and gas after 48 hours incubation in lactose McConkey broth was relied upon. It was of course only necessary to test the water as a routine after filtration and after chlorination. The efficiency of the preliminary sedimentation was estimated by a transparency test in which a piece of bright metal was observed through columns of water of different depths. It will be noted that the bacteriological standard is below the usual civil one, but the freedom of the troops from water-borne disease will be accepted even by slow sand filtration enthusiasts as a most convincing proof of the safety of rapid filtration and chlorination methods of treatment. It should also be remembered that the comparative immunity from disease occurred in a sub-tropical climate amidst conditions—concentration of troops on dust swept areas and initially most impure water—most favourable to heavy incidence of water-borne diseases.

It is not the writer's intention to discuss the advantages and disadvantages of the method from the financial point of view. These are fully set out in the

reports of the Metropolitan Water Board and elsewhere. He wishes, however, to place on record the efficiency of a method over which there is much important controversy, and it has been tested, not by laboratory experience, but on such a vital matter as the water supply of an advancing army.

The supply from the filters at Kantara was originally intended to supply 500,000 gallons a day for a small force of three divisions detailed to recapture the Egyptian frontier towns of El Arish and Rafa, to which the water was pumped in stages through a twelve-inch main laid upon the desert sand. It proved so successful, however, that when the conquest of Palestine was contemplated, the pipe-line continued to follow the advancing troops and was subsequently laid on to the Gaza-Beersheba line, *a distance of 147 miles from the filter-plant* and 220 from the Nile.

The ancient prophecy, that when the waters of Egypt should flow into Palestine that country should be delivered, thus came to be fulfilled as a result of a world-wide war. At the same time, there was carried out physiological experiment on a vast scale which proved that rapid filtration and chlorination methods of treatment can render an initially highly polluted and dangerous water *safe* for human consumption, and which has substantiated one of the most modern contentions as to the purification of water supplies.

In conclusion, I have to thank Sir Alexander Houston for the interest he has shown in my work, and for the furnishing of the reports of the Metropolitan Water Board and other references. My thanks are also due to the several bacteriologists of the Kantara Military Laboratory who, during the three-and-a-half years under review, were always willing to render assistance in the examination of special samples; and to my friend, Captain Rupert Briercliffe, O.B.E., R.A.M.C., who was associated with me in the work, for placing at my disposal many records to supplement my own.



## THE CHEMOTACTIC EFFECT OF OSMOSIS UPON LEUCOCYTES<sup>1</sup>.

BY FRANK MALTANER AND ELLA HOPPE.

*(From the Division of Laboratories and Research, New York  
State Department of Health, Albany.)*

THIS paper presents the results of a preliminary study of conditions affecting the movement of leucocytes. It demonstrates experimental facts which confirm the assumptions that osmotic force present in aqueous solutions and the permeability of leucocytes for water are the factors responsible for the movement of these cells from place to place.

### I.

The action of soluble substances in solution upon the movements of unicellular plants and animals has been extensively investigated and a voluminous literature has accumulated upon the subject. Solutions of different chemical substances undoubtedly have a varying effect upon the movements of these cells. The exact nature of the chemical or physical action involved has not, however, been satisfactorily demonstrated.

Pfeffer, who was one of the earliest workers in this field, at first attributed the movements of certain one-celled plants to osmosis. He later, after Stahl (1884), concluded that the stimulus was chemical in origin and gave the phenomenon its present name of "chemotaxis." His conclusions were extensively corroborated by various workers, but the development of physical chemistry and its application to the study of biological phenomena have done much to diminish the significance of the experiments upon which these conclusions were based.

Jean Massart, in 1889, demonstrated that the movements of bacteria and other plant cells were affected by soluble substances in solution. He concluded that the effect of solutions of different substances varies with the molecular weight and chemical structure of the substance. He said, "The repulsion exerted by these solutions is inversely proportional to the molecular weight and directly proportional to the isotonic coefficient. The substances which form exceptions are those which easily penetrate the cells in question." The conclusions of this author apparently show a direct connection between the molecular force which causes diffusion and the movement of the cells

<sup>1</sup> Read by title at the 108th meeting of the Society for Experimental Biology and Medicine, New Haven, Conn., May 22, 1920.

he studied. The proportionality to the isotonic coefficient also shows this motion is modified by the permeability of the cell membrane.

Several physical hypotheses have been developed with regard to the activities of amoebae. Certain of these which appear to be most accepted at present, have for their basis physical phenomena observed in a study of artificial amoebae. These artificial amoebae are drops of liquid suspended in another liquid in which they are insoluble. By producing local variations in surface tension at the surface layer of such a droplet it may be made to move in a manner strikingly similar to movements observed with the living amoebae.

While the analogy between the phenomena thus produced is a close one, the conditions under which they are observed are in fact quite dissimilar. A drop of liquid suspended in another liquid in which it is insoluble, can hardly be likened to a cell which is made up mostly of water and suspended in the same fluid. Local variations in surface tension and consequent formation of pseudopodia are haphazard and due to slight variations in tension at points upon the surface layer of the droplets. Even though these variations in surface tension occurring within the surface layers of the liquid droplets do account for tactile movements in living cells, it is unlikely that these forces either have sufficient magnitude or proper application to account for such motion as is indicated by the migration of leucocytes toward an infected area. In order to produce translation for a considerable distance in one direction, a sustained force of considerable magnitude is necessary in order to overcome the inertia of the liquid droplet or cell and the resistance offered by the liquid in which it is suspended and the surfaces with which it may be in contact. A careful analysis of these surface tension hypotheses has been made by Jennings (1915) with regard to the activities of amoebae and by Wells (1918) with regard to the movement of leucocytes.

If the experimental facts, based upon actual work with leucocytes, alone are considered, precedence must be given to the hypothesis that chemical affinity is the source of the stimulus which either produces or induces movements of translation of leucocytes. Among the most important studies upon this phase of the subject were those of Buchner (1890), Massart and Bordet (1891) and Gabritschewsky (1890). These workers used glass capillaries which were filled with solutions of the substances to be tested. They were sealed at one end and placed under the skin or in the peritoneal cavity of experimental animals. After several hours the capillaries were removed, washed, and examined as to their content of leucocytes. The presence of leucocytes within the tubes was regarded as indicating a positive chemotaxis and their absence a neutral condition or repulsion. By the use of this technique a great variety of substances were tested, only a few of which showed a definite attraction of leucocytes. The best attraction was obtained with bacteria and their extracts when these were free from toxin. Solutions of peptone, urea, dextrin, glucose and a few others also attracted leucocytes, while alcohol, acids, alkalies and salts repelled them. Sicherer used a technique *in vitro* in which



the capillaries containing the test solutions were suspended, with their open end just under the surface of a suspension of leucocytes. His results were similar to those obtained by the workers mentioned above but they were criticized by Pfoehl (1898) who maintained that the chemical nature of the phenomenon was not proven, and on the contrary concluded that the apparent results obtained were due to reactions of a physical nature.

## II.

At the beginning of the present study the technique *in vitro* of Sicherer (1899) was adopted on account of its simplicity and the possibility it gives for studying the physical conditions present. The substances used as test solutions were bacterial toxins and antitoxins, normal animal sera, broth medium, potassium tartrate and sodium chloride representing neutral salts, citric acid, quinine hydrochloride, glycerine, alcohol and 0.85 per cent. sodium chloride, which latter was included as a control upon the solution in which the leucocytes were suspended. The purpose of the experiment was to determine the chemotactic properties of the different substances, first in the form of their most concentrated solutions and second of gradually decreasing concentrations of the same substances.

Leucocytes were obtained by intraperitoneal injection of aleuronat into guinea-pigs. The leucocytes were washed twice and suspended in physiological salt solution (0.85 per cent.). Capillary glass tubes were filled with the various increasing dilutions of the substances to be tested, sealed at one end, and placed open-end down into the saline suspension of leucocytes. The suspensions of leucocytes were contained in small agglutination tubes and were about 1 cm. deep. A separate tube was used for each capillary. A temperature of 37.5° C. was used throughout. After one hour the capillaries were examined with a microscope, the height to which the leucocytes had risen was measured and the tube broken and its contents stained with Wright's stain.

All of these substances except alcohol and physiological salt solution showed strong attraction for leucocytes as represented by an accumulation of leucocytes within the capillary tubes. This evidence of attraction disappeared gradually and with varying rapidity as the concentration of the test solutions decreased. An examination of the densities of those dilutions of the test solutions in which no leucocytes accumulated revealed the fact that these dilutions possessed either the same or a lesser density than was possessed by the suspension of leucocytes. On the other hand, in every case where an accumulation of leucocytes was found within the capillaries, the densities of the test solutions were greater than the density of the leucocyte suspension.

On account of the vertical position of the capillaries above the suspension of leucocytes, it was thought that the leucocytes in contact with the denser solution at the opening of the capillaries might possess an increased buoyancy and as a consequence rise into the capillaries under the influence of gravity. However, in no case did the leucocytes accumulate at the upper end of the capillaries as would be expected if this were the cause. Nevertheless some of the tests were repeated with the capillaries in a horizontal position.

Table I.

Experiments *in vitro* demonstrating the accumulation of leucocytes in capillaries containing solutions at higher density than the solution in which they were suspended, and their absence from capillaries containing these same solutions at the same or a lower density than the leucocyte suspension.

TEST SOLUTIONS	DILUTIONS IN CAPILLARIES											
	1-0	1-5	1-10	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	1-5120
*Potassium tartrate	... + + +	+ + +	+ + +	+ + +	+	-	-	-	-	-	-	-
*Citric acid	... + + +	+ + +	+ + +	+ +	+	-	-	-	-	-	-	-
*Quinine hydrochloride	... + + +	-	-	-	-	-	-	-	-	-	-	-
Normal rabbit serum	... + + +	+ + +	+ + +	+ +	+	-	-	-	-	-	-	-
Normal horse serum	... + + +	+ + +	+ + +	+ + +	+ +	+	-	-	-	-	-	-
Diphtheria antitoxin	... + + +	+ + +	+ + +	+ + +	+ +	+	-	-	-	-	-	-
Tetanus antitoxin	... + + +	+ + +	+ + +	+ + +	+ +	+	-	-	-	-	-	-
Diphtheria toxin	... + + +	+ +	+	-	-	-	-	-	-	-	-	-
Diphtheria toxin heated	+ + +	+ +	+	-	-	-	-	-	-	-	-	-
Tetanus toxin	... + +	+	-	-	-	-	-	-	-	-	-	-
Tetanus toxin heated	... + +	+	-	-	-	-	-	-	-	-	-	-
Broth used for toxin prod.	+ + +	+ +	-	-	-	-	-	-	-	-	-	-
Physiological salt	... -	-	-	-	-	-	-	-	-	-	-	-
*Sodium chloride	... + + +	+ + +	+ + +	+ +	-	-	-	-	-	-	-	-
Alcohol	... -	-	-	-	-	-	-	-	-	-	-	-
Glycerine	... + + +	+	-	-	-	-	-	-	-	-	-	-

+ + + = More than 19 mm. rise.      + + = More than 4 mm. rise.      + = More than 1 mm. rise.

\* Dilutions of potassium tartrate, citric acid, quinine hydrochloride and sodium chloride were made up from saturated solutions.  
In thermostat for one hour at 37·5° C.  
Leucocytes suspended in 0·85 per cent. NaCl.

A cell was constructed about 18 mm. square and 5 mm. deep. The bottom of the cell was a three inch glass slide. The top was an inch cover-slip and the sides were built up with paraffin. A single capillary containing the test solution was thrust through a hole in the paraffin wall and parallel to the glass slide which formed the base. This was sealed in with a hot needle and the leucocyte suspension filled into the cell. The cover-slip was now sealed in place and the whole observed under the microscope in a 37·5° C. thermostat.

These tests gave results identical with those observed in the preceding experiment. The leucocytes accumulated only in those tubes which contained solutions possessing a greater density than that of the leucocyte suspension.

In order to more fully establish the effect of difference of density upon the movement of leucocytes a greater variety of substances were tested especially including several which according to previous investigators possessed positively chemotactic properties. Bacterial suspensions and extracts, starch, aleuronat, dextrose, lactose and other substances were used. Solutions or suspensions were prepared and their density adjusted to the same density as that of the suspension of leucocytes. Two groups of tests were performed, one in which the density was that of 0·85 per cent. sodium chloride solution at 37·5° C. and the other in which the density was adjusted to that of guinea-pig serum at the same temperature.

Two floating equilibria were constructed. One was adjusted to equilibrium at a temperature of 37·5° C. in an 0·85 per cent. solution of sodium chloride. The other was adjusted



at the same temperature but in normal guinea-pig serum. These equilibria were small glass balloons weighted with mercury and adjusted by fusing glass to the tips until they would neither rise nor sink in the solutions at 37.5° C. Test solutions of the various substances were prepared and adjusted to the same density as the leucocyte suspensions by means of the equilibria. The test solutions were filled into glass capillaries and set up as in the first set of experiments. The tests were carried out in a 37.5° C. thermostat. The capillaries were examined from time to time during 22.5 hours to determine whether a rise of leucocytes into the capillary tubes occurred.

Nine substances, namely, echinacea, phytolacca, quinine hydrochloride, urea, citric acid, acetic acid, dextrose, lactose and sodium chloride, were prepared from saturated solutions of these substances, in concentrations varying between 1 in 50 to 1 in 1000. The density of each dilution was adjusted

Table II.

Experiments *in vitro* demonstrating the fact that when solutions to be tested for chemotactic action, contained in capillary tubes, are adjusted to the same density as the solution in which the leucocytes are suspended, no leucocytes accumulate within the capillaries.

TEST SOLUTIONS		DILUTIONS IN CAPILLARIES											
		1-50	1-50	1-50	1-100	1-100	1-100	1-500	1-500	1-500	1-1000	1-1000	1-1000
Echinacea	*	—	—	—	—	—	—	—	—	—	—	—	—
Phytolacca	*	—	—	—	—	—	—	—	—	—	—	—	—
Quinine hydrochloride	+	—	—	—	—	—	—	—	—	—	—	—	—
Urea	+	—	—	—	—	—	—	—	—	—	—	—	—
Citric acid	+	—	—	—	—	—	—	—	—	—	—	—	—
Acetic acid	+	—	—	—	—	—	—	—	—	—	—	—	—
Dextrose	+	—	—	—	—	—	—	—	—	—	—	—	—
Lactose	+	—	—	—	—	—	—	—	—	—	—	—	—
Sodium chloride	+	—	—	—	—	—	—	—	—	—	—	—	—
		Distilled water extract			Filtered suspension			Supernatant fluid			Diluted with H <sub>2</sub> O		
Aleuronat		...	...	...	—	—	—	...	...	...	...	...	...
Starch		...	...	...	...	...	...	—	—	—	...	...	...
Diphtheria toxin		...	...	...	...	...	...	...	...	...	—	—	—
<i>B. typhosus</i>		—	—	—	...	...	...	...	...	...	...	...	...
<i>Staph. albus</i>		—	—	—	...	...	...	...	...	...	...	...	...
<i>B. coli</i>		—	—	—	...	...	...	...	...	...	...	...	...
<i>Staph. aureus</i>		—	—	—	...	...	...	...	...	...	...	...	...
<i>B. pyocyaneus</i>		—	—	—	...	...	...	...	...	...	...	...	...
Normal horse serum		...	...	...	...	...	...	...	...	...	—	—	—
Normal rabbit serum		...	...	...	...	...	...	...	...	...	—	—	—
Tetanus antitoxin		...	...	...	...	...	...	...	...	...	—	—	—
Dysentery antitoxin		...	...	...	...	...	...	...	...	...	—	—	—
Diphtheria antitoxin		...	...	...	...	...	...	...	...	...	—	—	—

\* = made from approximate normal tinctures.

+ = made from saturated solutions.

Leucocytes suspended in 0.85 per cent. NaCl.

Test solutions are adjusted to the same density as 0.85 per cent. NaCl by means of the floating equilibrium

Test solutions made from saturated solutions adjusted by dilution with water or with saline solution.

Temperature of exp. 37.5° C. Time 3 to 22½ hours.

to the desired density by the addition of water or salt solution. The bacterial emulsions and extracts and the normal sera, toxins and antitoxins were used in their concentrated form, the adjustment being made as before with water or saline.

None of the capillaries during any period of the experiment, which was continued for twenty-two and one-half hours, showed an accumulation of leucocytes. In these tests the chemical nature of the test substance certainly does not affect the movement of leucocytes. In Table II are recorded only the results obtained with densities adjusted to the density of 0.85 per cent. sodium chloride solution. The results obtained with the densities adjusted to the density of normal guinea-pig serum were absolutely identical, and for that reason have not been tabulated.

In view of the facts determined by use of the above technique, some tests were carried out with capillary tubes containing the test solutions inserted subcutaneously in guinea-pigs. Four test substances were chosen, three of which, according to Buchner and Gabritschewsky, were positively chemotactic, namely urea, peptone and *B. pyocyaneus* protein; the other, sodium chloride, was negatively chemotactic. Solutions of three different densities of each substance were tested. The densities chosen were normal guinea-pig serum density, and densities greater and less than this.

An aqueous extract of *B. pyocyaneus*, an aqueous solution of urea, one of peptone and one of common salt were prepared. Three dilutions of each were adjusted by means of the floating equilibrium; one to guinea-pig serum density, one of greater and one of lesser density. These dilutions were filled into capillary tubes 1.5 mm. in diameter and 12 mm. long and inserted under the skin of the abdomen of guinea-pigs. After eight hours they were removed and the contents examined for leucocytes.

It was impossible to obtain reliable results with this technique. The glass capillaries were often broken and in other cases fibrin-like plugs closed the openings so that the leucocytes could not penetrate even if an attraction was exerted by the test solution. The results obtained with solutions of sodium chloride and urea in guinea-pig no. 1 and the results with peptone in guinea-pig no. 3 show definitely the greater accumulation of leucocytes in those capillaries containing the more concentrated solutions of these substances.

### III.

The irregularities encountered by use of the capillary tube technique in animals led us to repeat the work of Buchner (1890) with bacterial protein, which has been generally accepted as demonstrating the chemical nature of the chemotaxis of leucocytes.

*B. pyocyaneus* was cultivated on potato slants at room temperature for 72 hours. On account of the scanty growth, the organisms were later cultivated on beef infusion agar at 37.5° C. for 24 hours, there being no apparent difference in the bacterial extract obtained. The bacterial mass was scraped from the media and rubbed up in about 50 times its volume of 0.5 per cent. caustic soda solution. This was left at 6° C. for 18 hours, a clear solution resulting. This solution was made faintly acid with N/10 hydrochloric acid, and the volu-



Table III.

Guinea-pig experiments *in vitro* demonstrating the effect of varying the density of the test solutions within capillary tubes inserted subcutaneously in guinea-pigs and removed after 8 hours.

Content of capillary	Pig Number I	Pig Number II	Pig Number III	Pig Number IV
Salt solution				
1. Isotonic	+	Capillaries of salt solution lost and confused with urea. Two of the six were recovered but not identified	+	Two capillaries lost, third not identified
2. Serum density	++		- air bubble at entrance	
3. Saturated solution	+++		- " "	
Urea solution				
1. Isotonic	+	Ditto	-	+
2. Serum density	++		++	+
3. Saturated solution	+++		++	+++
Peptone solution				
1. Isotonic	-	+	+	+
2. Serum density	+ dense fibrin mass	+	++	+ dense fibrin mass
3. Saturated solution	++ capillary broken	++ capillary broken	+++	+++
<i>Cyanocobalamin</i> extract				
1. Less than serum density	+	-	Two capillaries lost,	+
2. Serum density*	++	+	third not identified	+ dense fibrin mass
3. Greater than serum density*	Capillary lost	+		+++

\* = Density adjusted by addition of salt solution.

- = No leucocytes present.

+ = Few leucocytes in open end of capillary.

++ = Leucocytes approximately 6 mm.

+++ = Leucocytes entire length of capillary—12 mm.

minous precipitate formed was subsequently separated by centrifugalization. This precipitate was rubbed up in a little water and a small quantity of 0.5 per cent. caustic soda solution added, just sufficient to bring it into solution. This solution was dark brown and clear and gelatinized in the cold as described by Buchner.

The dissolved protein was sealed in freshly drawn out spindle shaped glass tubes 5 cm. long and about 6 mm. wide at the middle, and sterilized by boiling in a water bath for one hour.

A space on a rabbit's back about 4 inches square was shaved and a little slit made through the skin. The capillaries could be easily pushed through this slit and moved forward about 25 cm. from the point of insertion and the tips broken off. Control tubes were filled with isotonic salt solution. These capillaries were left in place for from four to 48 hours. They were then removed and examined macroscopically and with a hand-lens. The tubes were then broken into sections and the contents of the sections examined with a dark field and by staining.

Contrary to the results reported by Buchner no leucocytes accumulated in any of the capillaries containing either bacterial protein or isotonic salt solution. At first what appeared to be a cloud of leucocytes was observed in the tubes, but a closer examination showed this to be a precipitate which formed in the protein regardless of whether the tubes were placed in the animal or simply in the incubator for a similar period of time. The particles of this precipitate were exactly the size and had the general appearance of leucocytes, but were much more highly illuminated in dark field preparations,

and staining and microscopic examination showed them to be semi-crystalline in nature. Even when stained with Wright's stain they resembled leucocytes. At the openings, and extending a millimeter or two into the capillaries, plugs of fibrin-like clot containing a few leucocytes were invariably formed, both in the tubes containing bacterial protein and in those containing isotonic salt solution.

It was thought that positive results might be obtained if the capillaries containing the bacterial protein were placed in the peritoneal cavity. For this reason similar capillaries were filled with the protein and placed in the peritoneal cavities of rabbits and guinea-pigs. In some instances aleuronat or distilled water was injected into the cavity from four to five hours before the tubes containing the test solutions were inserted so as to have a considerable quantity of leucocytes present. In no case was an accumulation of leucocytes found within the capillaries other than the small plug at the opening and this was almost invariably present.

These same or similar plugs were observed by Buchner who seemed to regard them with the few leucocytes they contained as evidence of chemotaxis. He observed these plugs however only with positively chemotactic substances while our experiments showed them to be present regardless of the nature of the test solution.

#### IV.

As a result of our work with a capillary tube technique in animal experiments it was concluded that such a procedure was unsuitable for a study of the effect of substances in solution upon the movement of leucocytes, and that results by its use should be regarded with considerable scepticism. The experiments, on the other hand, by the use of capillaries *in vitro* gave what seemed to be reliable results and definitely indicated that the movement of leucocytes was due to some physical property which depended upon a difference in density between two solutions in contact with each other.

The early observations of Massart (1889), previously mentioned, regarding the effect of dissolved substances upon the movement of certain bacteria and plant cells, indicated that diffusing substances had a repelling action upon these cells and that this repulsion was in some manner dependent upon the isotonic coefficient. In other words, diffusion and the permeability of the bacteria for the diffusing substance were responsible somehow for their repulsion from the test solutions.

Now diffusion obviously occurs in solutions of different density in contact with each other, and especially when the solvent is the same for each solution. This diffusion takes place in two directions, the dissolved substances diffusing in one direction and the solvent in the opposite direction.

Animal and vegetable cells are in all cases easily permeable to water, while their permeability for dissolved substances differs considerably although it is always less than their permeability for water.



With these facts in mind the following conception was formulated as defining the conditions to be observed in a study of the action of soluble substances in solution upon the movement of leucocytes.

(1) That there is no inherent force within the leucocyte which can cause it to move considerable distances in a short space of time.

(2) That the force which produces this motion resides in the solution and is physical and not chemical in nature.

(3) That in a solution in which the solute is not at concentration equilibrium with its solvent, there exists a force of diffusion both upon the molecules of the solute and of the solvent.

(4) That the movement of leucocytes is from a less dense to a denser solution or from one of lesser to one of greater concentration in an aqueous medium and that this motion is due to the force of diffusion of the water molecules moving in a direction opposite to the force upon the molecules of solute.

(5) That a leucocyte is a particle of semi-permeable material which has taken up the solvent water in an amount far in excess of its mass as represented by protein and, as a consequence, moves by reason of the force of diffusion upon the molecules of water and in a direction opposite to the direction of action of the diffusing molecules of solute.

With this conception of the problem in mind the four following experiments were carried out.

#### TEST No. 1.

The first test shows that an artificial leucocyte which consisted of a cell in the shape of a sphere made of collodion and filled with water moves in water toward a source of diffusing saccharose.

A copper sterilizing pan five inches wide and one and one-half inches deep and ten inches long was divided into three compartments by means of parchment partitions. One of the end compartments was filled with a concentrated solution of saccharose and the other two with distilled water. A spherical sack of collodion one inch in diameter was filled with water, the opening tied with a silk thread and placed in the middle compartment.

After about 30 minutes this cell had moved over against the partition through which the saccharose was diffusing, and this occurred repeatedly during the course of the experiment regardless of where the cell was placed within the middle compartment.

#### TEST No. 2.

This test demonstrates the movement of leucocytes over a considerable distance toward a source of diffusing sodium chloride.

Ordinary six by five-eighths inch test tubes were drawn out at one end to a diameter of about 6 mm. They were bent to an angle of about 120 degrees at the beginning of the constriction. The constricted end was cut off about four inches from the bend.

Five batches of 1/2 per cent. aqueous agar were prepared containing respectively no sodium chloride, 0.5, 1, 2, and 3 per cent. sodium chloride.

The agar was then filled into the bulbs of the test tubes described and allowed to gelatinize. Washed guinea-pig leucocytes were suspended in 0.5 per cent. sodium chloride solution contained in a small crystallizing dish. The drawn out ends of the tubes were now filled with 0.5 per cent. saline and the open ends immersed in the suspension of leucocytes. The experiment was carried out in a thermostat at 37.5° C.

After four or more hours the leucocytes were found to have moved into the tubes containing the 2 and 3 per cent. sodium chloride for from three to four inches, while in the other tubes no leucocytes had accumulated.

#### TEST No. 3.

The following test is an actual demonstration of the presence of osmotic force and shows the effect of the diffusion of sodium chloride upon the rate of settling of leucocytes from a suspension.

A suspension of guinea-pig leucocytes was made in 0.5 per cent. sodium chloride solution. This was filled into two small test tubes, about 2 c.c. of suspension in each. At the surface of the suspension in each tube there was placed by means of wire loops a small particle of agar (about 3 mm. cross section). This agar was plain aqueous 1 per cent. agar in one tube and in the other was 1 per cent. agar containing 3 per cent. sodium chloride. These tubes were placed in the thermostat at 37.5° C.

The suspension contained in the tube with the plain aqueous agar settled rapidly to the bottom while that contained in the other tube had not settled in five hours.

This experiment was repeated using pellets of agar in place of the particles described above. These pellets rested upon constrictions made in the test tubes. The results were similar.

#### TEST No. 4.

The last test shows that an insoluble substance like casein has no effect upon the rate of settling of leucocytes from a suspension, but that when a soluble substance is produced by tryptic digestion, the same effect is observed as with sodium chloride.

This experiment was set up in the same manner as test no. 3, using two test tubes containing the leucocyte suspension. The agar pellets, however, contained in the one case powdered casein and in the other a mixture of casein and trypsin.

The leucocytes in the tubes containing the casein agar pellet settled quite rapidly to the bottom, while in the tube containing the casein trypsin agar mixture, the leucocytes remained suspended for more than six hours.

Regardless of any theoretical considerations these four experiments together with capillary tube experiments *in vitro* demonstrate that with the substances used, and in an aqueous medium, leucocytes move in a direction opposite to that in which the dissolved substances are diffusing. This supports the hypothesis that the movement of leucocytes is due to the force of osmosis which exists in a solution not at concentration equilibrium, and that the application of that force depends upon the greater permeability of leucocytes for water than for the dissolved substances.



## REFERENCES.

- BUCHNER, H. (1890). Die Chemische Reizbarkeit der Leukocyten und deren Beziehung zur Entzündung und Eiterung. *Berlin. klin. Wochenschr.* xxvii. 673.
- (1890). Ueber pyogene Stoffe in der Bakterienzelle. *Ibid.* p. 1084.
- GABRITSCHESKY, G. (1890). Sur les propriétés chimiotactiques des leucocytes. *Ann. Inst. Pasteur*, iv. 346.
- JENNINGS, H. S. (1915). *Behaviour of the lower organism*. New York: Columbia Univ. Press. (Columbia University Biological Series, X.)
- MASSART, J. (1889). Sensibilité et adaptation des organismes à la concentration des solutions salines. *Arch. de Biol.* ix. 515.
- MASSART, J. and BORDET, J. (1891). Le chimiotaxisme des leucocytes et l'infection microbienne. *Ann. Inst. Pasteur*, v. 417.
- PFEFFER, W. (1900-06). *The Physiology of Plants*, tr. and ed. by A. J. Ewart, 3 vol. Ed. 2. Oxford: Clarendon Press.
- PFOEHL, J. (1898). Chemotaxis der Leukocyten in vitro. *Centralbl. f. Bakteriöl. etc.*, xxiv. 343.
- SICHERER, O. (1899). Zur Chemotaxis der Leukocyten in vitro. *Ibid.* xxvi. 360.
- STAHL, E. (1884). Zur Biologie der Myxomyceten. *Botanische Zeitung*, xlii. 144, 160, 187.
- WELLS, H. G. (1918). *Chemical Pathology*. Ed. 3.

MALARIA IN ENGLAND

WITH SPECIAL REFERENCE TO THE ROLE OF TEMPERATURE  
AND HUMIDITY.

BY BREVET LIEUT.-COLONEL C. A. GILL, I.M.S., D.P.H., D.T.M. & H.  
*Chief Malaria Medical Officer, Punjab.*

(With one Map.)

CONTENTS.

	PAGE
I. Preliminary Considerations . . . . .	320
II. The Seasonal Incidence of Endemic Malaria in England . . .	321
III. The Recent Distribution of Endemic Malaria in England . . .	323
IV. The Past History of Endemic Malaria in England . . . . .	328
V. Conclusion . . . . .	330
Summary . . . . .	332

I. PRELIMINARY CONSIDERATIONS.

THE writer has recently been engaged in India in the study of the part played by meteorology in malaria and in a forthcoming paper a detailed account of the investigation, so far as it has at present gone, is recorded<sup>1</sup>.

This communication is solely concerned with the application to English conditions of the following conclusions which are thought to throw light on the malaria problem in England.

(1) Meteorological factors, more particularly the temperature and humidity factors, play an important part in malaria since they are largely concerned in determining the conditions necessary for the transmission of infection.

(2) In any attempt to determine the influence of either temperature or humidity in malaria it is necessary to take into account both the temperature and the humidity factors.

(3) The transmission of malaria in India is mainly confined to periods when the temperature and humidity factors are above a definite height, the lower limits of which are approximately represented by a monthly mean temperature of 61° F. and a monthly mean relative humidity of 63 per cent. (as measured at 8 a.m.).

(4) When both factors are above the “critical” figure a period of potential infection occurs and when either factor is below the “critical” figure a period of interrupted infection supervenes.

(5) Provided both factors are above the “critical” figure, a high mean temperature and a high mean humidity are more favourable to the transmission of malaria than a low mean temperature and a low mean humidity.

<sup>1</sup> Gill, C. A. (x. 1920), The Rôle of Meteorology in Malaria, *Indian Journ. Med. Research*.



It was also pointed out that in Northern India, where the mean temperature during the greater portion of the year is uniformly above 61° F., the humidity factor, by reason of its marked seasonal variability, exercises a predominant part in determining the season of the year and the duration of the period during which the transmission of infection may take place.

In temperate climates, on the other hand, where a high degree of atmospheric humidity more constantly prevails and where the mean temperature during the hottest months is in the vicinity of the "critical" temperature (61° F.), it was surmised that the temperature factor would be likely to be of major importance in determining the occurrence of periods of potential infection.

In these circumstances it was decided to examine the temperature and humidity conditions prevailing in the United Kingdom with a view to ascertaining whether they confirmed or rebutted the conclusions detailed in the above summary.

As the result of applying this test has afforded evidence suggesting that the temperature and humidity factors play an important part in determining the incidence and distribution of indigenous malaria in England, and that by taking into account the influence exerted by these factors some obscure points in the endemology of malaria may be explained, it has been thought expedient to consider the subject in some detail.

I desire to take this opportunity of recording my gratitude to the Hon. Major-General W. R. Edwards, C.B., C.M.G., M.D., Director-General, Indian Medical Service, for permitting the publication of this note in England, and also for the encouragement which his interest in the main investigation has occasioned.

## II. THE SEASONAL INCIDENCE OF ENDEMIC MALARIA IN ENGLAND.

The first point it was sought to determine has reference to the season of the year in which it is held that meteorological circumstances in the United Kingdom permit of *infection* taking place, which, of course, must be clearly distinguished from the season of the year in which *attacks* of malaria may occur.

Applying to the United Kingdom the conclusion that a mean monthly temperature of not less than 61° F. approximately represents the lower limit of temperature necessary to create a period of potential infection, it is found from a scrutiny of *The Book of Normals*<sup>1</sup> that in respect of the 164 recording stations in the United Kingdom (Scilly and the Channel Islands being excluded), the mean monthly temperature in no part of the kingdom reaches 61° F. except during the months of July and August. There is one exception to this rule, viz. Camden Square, London, N.W., where the mean temperature in June is 61° F. (It is however stated that the thermometer at Camden Square

<sup>1</sup> *The Book of Normals of Meteorological Elements for the British Isles for periods ending 1915.* The Meteorological Office, Kingsway, London, W.C.

is not kept, as elsewhere, in a Stevenson screen and it is thus not protected from indirect radiation.)

A further analysis of the temperature conditions in July and August shows that as a general rule, to which there are a few exceptions, the mean temperature is slightly higher in July than in August. It is also clear that these two months are appreciably hotter than any others, so that under normal conditions it would not be anticipated that the temperature factor would be suitable to the transmission of infection during any other month in the year.

In view of this conclusion, it is of interest to note, in the Reports on Malaria, published by the Local Government Board and the Ministry of Health<sup>1</sup>, that the majority of the indigenous cases of malaria recently reported in England have been considered, on clinical and epidemiological grounds, to have been acquired during the months of July or August. A certain number of primary attacks have been attributed to infections acquired in the spring, but it is for consideration whether these cases are not examples, as some authorities are inclined to think, of the tardy appearance of infections acquired in the previous year.

In regard to relative humidity the mean figures for a large number of areas are not available, but I have been referred by the Director of the Meteorological Office, to a paper by W. F. Stacey (1915)<sup>2</sup>, from which it appears that the monthly mean relative humidity (at 9 a.m.) in 91 recording stations during the period 1901–1910 never falls below 70 per cent. during any month of the year.

In connexion with the use of monthly mean figures of meteorological elements for the purpose of determining periods of potential infection it is necessary to emphasise certain points.

In the first place it is possible that the meteorological data may not accurately reflect the climatic conditions in every part in each meteorological area, nevertheless, until the contrary be proved, it seems permissible to assume that they do.

Secondly, it is recognised that monthly mean figures do not form entirely satisfactory data, since they fail to take into account the conditions prevailing in exceptional years, whilst they may refer to widely different conditions.

Monthly mean figures therefore form no substitute for, and do not *necessarily* give any indication concerning, the actual conditions of temperature and humidity associated with the transmission of malaria in England, but until the latter have been determined it is thought that the monthly mean figures may be made to serve a useful purpose.

<sup>1</sup> James, S. P., Reports and Papers on Malaria contracted in England in 1918, *Report to the Local Government Board on Public Health and Medical Subjects*, New Series, No. 123. See also *Malaria at Home and Abroad* by the same author. John Bale, Sons and Danielsson, Ltd. London 1920.

<sup>2</sup> Stacey, W. F. (1. 1915), Distribution of Relative Humidity in England and Wales, *Quart. Journ. Royal Meteorol. Soc.* xli, No. 173.



The following conclusions are drawn in regard to the influence of temperature and humidity on the seasonal incidence of malarial infection in the United Kingdom:

1. Relative humidity being at all times favourable plays little or no part in determining the seasonal incidence of malarial infection in England.

2. The temperature factor exercises an important rôle since it limits the period during which malaria may ordinarily be acquired in the United Kingdom to the months of July and August.

### III. THE RECENT DISTRIBUTION OF ENDEMIC MALARIA IN ENGLAND.

It necessarily follows from the part attributed to meteorological factors in determining the required conditions for the transmission of malaria that the distribution of the endemic centres of malaria in the United Kingdom should conform to these conditions, and that, for reasons already stated, a predominant influence should be exerted by the temperature factor.

In order to test the accuracy of this view the accompanying Map 1 has been prepared in accordance with the following plan:

Areas	Mean temperature in areas during July and August	Shown in map
1	62° F. or over	Shaded vertically
2	61–62° F.	Stippled
3	60–61° F.	Shaded obliquely
4	Under 60° F.	Blank

It will be seen that the distribution of temperature during the hottest months in the year (as measured by monthly mean temperature) is in some respects peculiar, and differs from the distribution which on general grounds might have been anticipated.

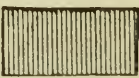


The area showing a mean temperature during July and August of 62° F. or over, which is extremely small in size, comprises a strip of country in Essex and Kent on each side of the estuary of the Thames and the lower portion of the Thames valley. *Area 1* also includes a strip of country in the south of Hampshire in the vicinity of the New Forest together with the eastern portion of the Isle of Wight. The towns of Brighton and Clifton also belong to *Area 1*.

*Area 2*, which is much larger than *Area 1*, is bounded on the south by the coast-line from the vicinity of Dover to the west of Bournemouth, whilst its northern boundary is formed by a line which runs more or less obliquely across England from a point on the east coast of Norfolk to the Bristol Channel in the vicinity of Cardiff.

Its western boundary is formed by a line which first runs due north from the vicinity of Bournemouth, and then turns west and running parallel to the Bristol Channel, ends on the coast in the neighbourhood of Ilfracombe. Small areas surrounding Torquay, Teignmouth and Plymouth also belong to *Area 2*.



Explanation.

Zone with mean temperature in July—August	over 62° F. =	
	61° F.—62° F. =	
	60° F.—61° F. =	
	under 60° F. =	unshaded
Ancient distribution of endemic malaria		= x
Recent distribution (1917—1919) of endemic malaria		= •

NOTE:—A case of malignant tertian malaria believed to have been contracted at Liverpool was reported in *The British Medical Journal*, Nov. 27, 1920. The patient—a girl of 18 years of age—was born in Liverpool and lived there for 10 years, but it is not clear where she lived during the remaining eight years nor whether she always resided in Liverpool during July and August. The proximity of Liverpool to Area 3 may also be noted.



*Area 3* consists of a strip of country lying to the north of and parallel to *Area 2* except in the east, where its northern boundary diverges so as to include an area in the south of Yorkshire. The estuary of the Dee and a small area along the north coast of Wales also belong to this area, whilst in England an area around Shrewsbury, the south coast of Devon and three small coastal areas in Cornwall all belong to *Area 3*.

*Area 4*. The rest of England and Wales, with the exception of the city of Manchester and the town of Aberdovey in Wales, the whole of Scotland and the whole of Ireland (excluding the city of Dublin) all belong to *Area 4*, in which the mean monthly temperature during July and August—the hottest months—never normally reaches 60° F.

It is now necessary to determine the relationship of these four areas to the present-day distribution of endemic foci of malaria.

From the reports issued by the Local Government Board—now the Ministry of Health—it appears that during the period 1917–1918 382 indigenous cases of malaria were discovered in 27 different localities in the United Kingdom. Lieut.-Colonel S. P. James, M.D., I.M.S. of the Ministry of Health, has kindly supplied me with the figures for the year 1919 during which 92 additional cases were discovered amongst the civil population and 13 fresh endemic foci. Reliable figures are therefore available regarding 40 endemic foci and 404 indigenous cases of malaria.

If the localities and the number of cases occurring in each be allotted to their respective areas (see Map, p. 324) the result shown in Table 1 is obtained.

Table 1.

The Distribution of Endemic Malaria in the United Kingdom by Areas.

Area	County	Place	No. of Cases	Remarks
Area 1	Kent	Sheppey and <i>Sheerness</i> *	172	Sea-level (approx.).
		<i>Isle of Grain</i>	59	„ „
		Sandwich	78	7 feet
		<i>Queenborough</i>	23	Sea-level (approx.)
		Chatham	2	8 feet
		Sittingbourne	3	4 feet
		Iwade	1	32 feet
		Minster	1	Sea-level (approx.)
	Essex	<i>Tilbury</i>	3	Sea-level (approx.)
		Purfleet	1	31 feet
	Middlesex	Walton on Thames	2	40 feet
	Hampshire	<i>Portsmouth</i>	1	Sea-level (approx.)
		<i>Southampton</i>	1	„ „
		<i>Emsworth</i>	1	„ „
Area 2	Essex	<i>Bournes Green</i>	2	90 feet
		<i>Walton on Naze</i>	7	Sea-level (approx.)
		<i>Dovercourt</i>	6	10 feet
		<i>Shotley</i>	1	Sea-level (approx.)
		<i>Brightlingsea</i>	1	84 feet
		Colchester	2	82 feet, R. Colne

\* Localities italicised are situated on the coast.

Table 1—*contd.*

Area	County	Place	No. of Cases	Remarks
Area 2	Suffolk	<i>Felirstowe</i>	2	Sea-level (approx.)
		<i>Bawdsey</i>	3	„ „
		<i>Southwold</i>	1	„ „
	Norfolk	Thetford	2	35 feet, R. Little Ouse
	Huntingdon	St Ives	1	27 feet, R. Ouse
		Fletton	1	27 feet, R. Nen
	Hertford	London Colney	1	225 feet, R. Colne
	Kent	Lydd	3	10 feet
	Sussex	Uckfield	1	43 feet, R. Ouse, Sussex
	Hampshire	Aldershot	9	256 feet
Area 3	Dorset	<i>Parkstone</i>	1	Sea-level (approx.)
		Shrewsbury	1	193 feet, R. Severn
		Atcham	1	149 feet, R. Severn
		Coalport	3	200 feet, R. Severn
		Leicester	1	200 feet
	Norfolk	Fakenham	2	115 feet, R. Wensum
	Lincoln	Grantham	2	162 feet, R. Witham
		<i>Tetney Lock</i>	1	Sea-level (approx.)
	Nottingham	Chilwell	1	18 feet
Area 4	—	—	—	—

A summary of the above figures gives the result shown in the following table.

Table 2.

Areas	Endemic localities		Cases of malaria	
	Actual	Percentage	Actual	Percentage
Area 1	14	40·0	350	86·6
Area 2	17	40·0	42	10·4
Area 3	8	20·0	12	3·0
Area 4	0	0·0	0	0·0
Total	39	100·0	404	100·0

It is thus seen that 40 per cent. of the endemic localities and no less than 86 per cent. of the cases are included in the extremely small zone in which the mean temperature during July and August—the hottest months—is over 62° F., while 40 per cent. are scattered in a much larger area in which the mean July-August temperature is between 61° F.–62° F.

There are eight localities in the zone exhibiting a mean temperature during July and August of 60° F.–61° F., whilst no endemic localities exist in the rest of the United Kingdom, in which the mean temperature during the hottest months (July–August) is below 60° F.

A consideration of the distribution of mean temperature in the United Kingdom therefore appears to suggest that the temperature factor may play an important part in determining the distribution and incidence of endemic malaria in this country.

But the temperature factor alone does not afford an explanation of the peculiar distribution of the endemic localities *within the above zones* and it



is thought that by taking into account the humidity conditions some light may be thrown on this point.

Mr Stacey shows (*loc. cit.*) that whilst at no season of the year does the mean monthly relative humidity (as measured at 9 a.m.) fall below 70 per cent., the areas showing the *lowest* mean relative humidity in July and August correspond approximately with the areas exhibiting the *highest* mean temperature.

It is clear therefore that little or no relationship exists between the areas showing relatively high degrees of humidity during the summer and the geographical distribution of endemic malaria; but this lack of correlation would be anticipated, since in many of the areas exhibiting relatively high degrees of humidity, the temperature factor is not favourable to the transmission of malaria.

It is clearly necessary to consider the distribution of the endemic centres *within the area exhibiting a suitable mean temperature* in relation to their mean relative humidity.

Unfortunately so little is known in regard to relative humidity that no precise indications of its distribution can be given, but some conclusions can be reached as the result of a consideration of the following circumstances. In the first place it may be assumed that the atmosphere in low-lying areas, if the latter are situated on the sea-coast or in badly drained riverain tracts, in addition to being relatively warm, will also be relatively humid. Secondly, Mr Stacey gives as one of his four general conclusions that "In summer the air over the interior of the country (England and Wales) is drier than over the coastal regions."

It will be seen from Table 1 (pp. 325, 326) that, of the 39 endemic localities in England, 17 are situated on the coast (names italicised in the Table), whilst, if those which are less than 100 feet above sea-level and are at the same time in close proximity to the coast, be included no less than 26 or 65 per cent. of the endemic localities exhibit the climatic conditions prevailing in coastal regions during the summer.

Of the remaining (inland) endemic centres it is noticeable that they are all located in low-lying situations—none being above 300 feet above sea-level—and they are in close relation with river valleys or marsh land. Thus three are situated in the Fen district, three in the Severn valley and three in the Thames valley.

Whilst therefore no detailed consideration of this subject can be given, the facts suggest that the precise location of the endemic centres of malaria in England, *within the area exhibiting a favourable mean temperature*, may be partly explained by the rôle played by relative humidity.

## IV. THE PAST HISTORY OF MALARIA IN ENGLAND.

The bearing of the conclusions regarding the rôle attributed to temperature and humidity in determining the recent distribution of endemic malaria may with advantage be considered in relation to its former distribution in the United Kingdom.

Owing however to the imperfect knowledge prevailing in regard to "fevers" in general (and malaria in particular) up to quite recent times, the past history of malaria is wrapped in obscurity. Nuttall, Cobbett and Strangeways-Pigg (1901)<sup>1</sup> give a detailed account, so far as it could be ascertained, of the conditions prevailing chiefly in the 18th and 19th centuries, and from the map accompanying their paper the endemic localities existing at this period have been added to my Map (p. 324), where they are shown as crosses.

It will be seen that the Thames valley and estuary of the Medway in *Area 1* were, as now, relatively severely infected, and that in the Fen district many centres of endemic malaria, which are only represented at the present time by St Ives and Fletton, existed.

There is also in *Area 2* an endemic focus in the vicinity of the Romney marshes, in the neighbourhood of which one endemic centre—Lydd—has recently been detected. Another small endemic area existed near the mouth of the Ouse river in Sussex, whilst recently one indigenous case has been reported from this neighbourhood (Uckfield).

Endemic areas in Hampshire were Lymington, Christchurch, and Lyndhurst, which are again represented by the indigenous cases recently reported from Southampton, Parkstone, Portsmouth and Emsworth.

In *Area 2*, moreover, an endemic centre existed near Bridgwater, where in four villages at the mouth of the Parrett river, malaria was formerly prevalent. No cases have recently been reported from this area, but Lieut.-Colonel James informs me that one case has been reported from Bridgwater, which he did not regard as being of undoubted indigenous origin as the patient had been in north Russia a year previously.

In *Area 3* there were some 16 endemic localities, mostly in Norfolk and Lincoln which are represented in 1917–1919 by Grantham and Tetney Lock in Lincoln and Fakenham, Thetford in Norfolk and Southwold in Suffolk.

Finally in *Area 4*, in which it would not be anticipated that any endemic localities would occur, except for two places on the Yorkshire coast—Partrington near Spurn Head and Hull, which are on the coast and almost within *Area 3*—the only endemic areas in *Area 4* were Carlisle in Cumberland, Kendal and Ulverston in Westmoreland, Garstang and Kirkham in Lancashire and Penzance in Cornwall.

<sup>1</sup> Nuttall, G. H. F., Cobbett, L., Strangeways-Pigg, T. (1901), *Studies in Relation to Malaria. The geographical distribution of Anopheles in relation to the former distribution of ague in England*, *Journ. of Hygiene*, 1, 4–44, 2 maps.



In regard to these areas Nuttall, Cobbett and Strangeways-Pigg (*loc. cit.*) give the following information on the authority of the observers quoted:

- (1) Carlisle: "Ague not endemic. In 1859 of 2580 patients treated at the dispensary only three entered as suffering from intermittent disease" (Whitley 1864).
- (2) Kendal: "From 1795-1821 118 cases of intermittent disease occurred amongst 28,700 patients, but between 1813-1821 there were only six cases" (Proudfoot 1795-1821).
- (3) Ulverston: "No intermittent disease was seen during 30 years practice but he had heard that a good deal existed at the beginning of the century" (Dickenson).
- (4) Garstang: "Some intermittent disease in 1826-1827 but none since" (Bell 1826-1827).
- (5) Kirkham: Dr Gradwell reports seeing much ague prior to 1831.
- (6) Penzance: Only three cases in a period of 17 years amongst 8,800 patients (Forbes, 1796-1822).

The above authors were unable to obtain any information in regard to the occurrence of endemic malaria in Scotland. They state however that "on the borders of Scotland ague was formerly very prevalent, and very common about Berwick and at Roxborough in 1807."

In regard to Ireland they state that this country "has the reputation of being exempt from ague, the peat bogs being especially stated to be free from the disease, but Wylde speaks of the occurrence of ague in Ireland and of the occurrence of an epidemic in Dublin in 1805<sup>1</sup>."

It is considered that the above-quoted statements cannot be considered as proving the former existence of endemic malaria in either Scotland or Ireland and that it is open to doubt whether the north-west corner of England was ever malarious.

In the case of the south of England and the Midlands the evidence is, on the other hand, entirely convincing that the disease was in certain parts prevalent and relatively severe. Thus Nuttall, Cobbett and Strangeways-Pigg (*loc. cit.* p. 26) conclude that "In England malarial disease seems to have been endemic only in the low-lying, ill-drained swampy districts where there was abundance of stagnant or slowly-flowing shallow water. Among such places the principal were the Fens of Cambridgeshire, Lincolnshire and the surrounding counties, the marshes on either side of the estuary of the Thames in Kent and Essex, the marshes of Romney and Pevensey on the south coast and those around Bridgwater on the Bristol Channel."

The same authors conclude, as the result of a scrutiny of the death-rate from malaria during the years 1850-1858 in the Fen district and in Kent respectively, that the disease was more fatal at that time in the low-lying lands in Kent bordering on the estuary of the Thames than in the Fen country around Huntingdon and Wisbech. They add "And this is in substantial agreement with what Defoe wrote a century earlier."

<sup>1</sup> In a recent book, *The Influence of Man on Animal Life in Scotland*, Professor Ritchie states that malaria was formerly prevalent in parts of Scotland and he shows on a map the location of the endemic localities. Its seasonal incidence is stated to have been vernal rather than autumnal and its disappearance is said in some cases to have followed the cessation of emigration of agricultural labourers from malarious parts of England.

This brief and incomplete review of the past history of malaria in the United Kingdom therefore suggests that, whilst it has during recent years become greatly reduced in incidence and severity, its distribution has not undergone any material change.

It is therefore concluded that the view reached in regard to the part played by temperature and humidity is not rendered untenable by any facts which are forthcoming as the result of a scrutiny of the past history of the disease.

#### V. CONCLUSION.

It is thought that the adoption of the view attributing an important part to temperature and humidity in determining the distribution of malaria in England affords an explanation of some obscure points in connexion with the endemiology of the disease.

Lieut.-Colonel S. P. James, M.D., I.M.S., draws attention (*loc. cit.*) to one of these points when, in connexion with his recent work in England, he states:

“It was found:

- (1) That in a number of places which harboured many carriers and numerous anophelines no new cases of malaria arose.
- (2) That some of the new cases recorded arose in areas which harboured only one or two carriers and exceedingly few anophelines.”

“The occurrence, in England,” he adds, “of these two conditions (sometimes termed ‘Paludism *sine* Malaria’ and ‘Malaria *sine* Paludism’ respectively) is of great interest. The problem of defining the particular localities and circumstances in which we may expect an origin of new cases and a spread of malaria would be solved if we could explain these occurrences correctly.”

It is thought that in the light of the part ascribed to the temperature and humidity factors an explanation of these two conditions is forthcoming. Thus it is clear, in the case of England, that if the temperature during July and August is unfavourable to the transmission of malaria, the local incidence of the disease will not be affected by the presence of many human carriers and numerous anophelines (Paludism *sine* Malaria).

On the other hand, in areas where the temperature and humidity factors are favourable, the transmission of infection will be apt to occur provided one or two carriers are present together with relatively few anophelines (Malaria *sine* Paludism).

In these circumstances it would not be anticipated that any exact correlation would exist between the distribution of carrier species of anophelines and the distribution of endemic malaria in the United Kingdom, and it is now known that these insects prevail in many parts of England without giving rise to indigenous cases of malaria.

It is possible that the part assigned to the temperature factor may to some degree explain the gradual decline of malaria in England.

The endemic area in this country is situated near the northern limit of



the area in which the mean temperature during the hottest months is favourable to the transmission of the disease. In this area a cold summer—and more especially a succession of cold summers—would be anticipated in the course of time to lead to a gradual decline in the incidence of the disease, for in such circumstances the “recovery-rate” would exceed the “infection-rate.”

An interruption of infection, as the result of unfavourable temperature conditions, would necessarily occur more frequently in the Fen district in the north than in Kent in the south, and it would therefore be anticipated (as indeed appears to be the case) that the decline of the disease would take place more rapidly in the former than in the latter area.

Finally, on this view, the main factor in determining an unusual prevalence or a mild epidemic in England would be an unusually hot summer.

In this connexion it is of interest to note that the last occasion on which malaria is known to have prevailed extensively in England was in the years 1858–1859. On consulting the meteorological report for 1858<sup>1</sup> it is found that this year was rendered noteworthy by reason of the exceptionally long and hot summer<sup>2</sup>. The mean temperature in June was extraordinarily high (64·9° F. as compared with a mean of 59·4° F. at Greenwich), and the Registrar-General in his report for the year<sup>3</sup> states: “The heat in June was so great that there was no instance except one since 1771 in which the mean temperature for that month has been exceeded<sup>4</sup>.”

It was thought that slight but progressive changes in climate, involving a reduction in the mean temperature during the summer, might also form a contributory factor to the decline of malaria in England, but the meteorological authorities have no knowledge of any secular changes in climate; and, indeed, it is not necessary to postulate their occurrence, in view of the fact that normal oscillations in the climatic conditions prevailing in England suffice to determine a considerable amount of interruption of infection in certain years.

No doubt other circumstances have contributed to the spontaneous decline of malaria in England. Thus the drainage of water-logged areas, and more especially the elaborate system of drainage carried out in the Fen district, must not only have considerably reduced the facilities for the breeding of anophelines, but in addition may have rendered the humidity conditions less favourable than formerly to the transmission of infection.

The intensive cultivation of the soil and the great extension of urban areas have also completely changed the environment in certain malarious tracts.

<sup>1</sup> *Quarterly Weather Reports*, England, 1858.

<sup>2</sup> It is assumed that the prevalence of malaria in 1859 was mainly the result of infections acquired during the previous year.

<sup>3</sup> *Report of the Registrar-General of Births and Deaths*, 1858.

<sup>4</sup> Since this communication was submitted for publication my attention has been called to a report by Angus Macdonald (*War Office Observations on Malaria*, December, 1909) in which the influence of temperature on malaria in England is clearly recognised and emphasised and detailed temperature statistics for the years 1858–1860 are given.

It is thus not difficult to understand the reason why Lambeth and Pimlico in London, which in the 18th century were notoriously malarious, are now free from the disease. Finally, in view of the part played by economic and social conditions in maintaining infection in malarious communities, it would be anticipated that the great amelioration in these conditions which has taken place, more especially during the past 100 years, must have been a further factor in favouring the spontaneous decline of the disease.

In view of the operation of all these factors—amongst which an important part is assigned to the meteorological factors—it is possible to offer an explanation of the gradual disappearance of malaria from England, and to hazard the opinion that the introduction from abroad of human carriers is not likely to interfere materially with its final extinction.

#### SUMMARY.

(1) The study of the meteorological circumstances prevailing in the United Kingdom confirms the conclusions reached in India that the combined influence of temperature and humidity plays an important part in determining the conditions necessary for the transmission of malaria.

(2) The part assigned to temperature and humidity points to the conclusion that the period of active infection in England is ordinarily limited to the months of July and August.

(3) The part attributed to temperature accounts for the geographical distribution of the endemic area of malaria in the British Isles and explains its limitation to certain parts of England.

(4) The combined influence of temperature and humidity appears to afford an explanation of the precise location of the endemic centres of malaria within the above area.

(5) The part assigned to temperature and humidity elucidates certain points in connexion with the endemiology of malaria in England, and it offers an explanation of the conditions known as "*Malaria sine* Paludism" and "*Paludism sine* Malaria."

(6) The part played by the meteorological factors throws light on the past history of malaria in England and it helps to explain its gradual decline.



## A VALUATION OF THE "AGGLUTINABILITY-FACTOR" IN DREYER'S SYSTEM.

By A. D. GARDNER, D.M. (Oxon.), F.R.C.S. (Eng.).

(From the Standards Laboratory, Department of Pathology, Oxford.)

ONE of the main postulates, upon which Dreyer's system for agglutination-tests is based, is that no two suspensions of any organism can be taken as identical in agglutinability. This fact is now too well proven to need further demonstration, though it is still disregarded in many a current method.

There are two ways of meeting the difficulty, and both have been adopted at different periods in the manufacture of sterilized cultures for the Widal test (see Dreyer, 1909).

(1) All suspensions are brought to one and the same degree of agglutinability by suitable additions of culture-filtrate, to reduce the sensitiveness, or of salt-solution, to increase it.

(2) Each suspension is diluted with salt-solution to a suitable constant density, and its sensitiveness is carefully estimated by comparison with an arbitrarily chosen "standard" suspension, or with any other suspension already standardised against the latter. The agglutinability of the new suspension in relation to the standard can then be expressed numerically in the form of an "Agglutinability factor."

This factor, and its operation, form the subject of the experiments described in this paper—experiments in which an attempt has been made to estimate to what extent the agglutinability-factor succeeds in reducing to manageable order the numerical chaos that results from the variability of suspensions of an agglutinable bacillus (*B. typhosus*).

To this end, a number of specimens of blood were obtained from patients suffering from typhoid fever, and from persons who had been inoculated with *B. typhosus*.

From the stock of "standard" cultures of *B. typhosus*, which have been issued from the Standards Laboratory at one time or another, five were selected which showed a considerable range of agglutinability (*i.e.* a range of more than 100 per cent. difference), were of widely different ages (see list below), and included at least one suspension of relatively inferior quality (*viz.* T 32 which, though quite fit for routine use, had always been regarded as somewhat hyper-sensitive, and as an inferior clumper).

Each serum was suitably diluted, after a rough preliminary test, and titrated at one and the same time against each of the suspensions employed.

Since the full technique has been described in several recent articles (*e.g.* Gardner, A. D., 1918) and is identical in all essentials with the original method used by Dreyer and his co-workers since 1905 (see Schroeder, 1909), a detailed description of it will be omitted.

The long series of 12 tubes was used, except on a few occasions (when insufficient serum was available). Drop measurement was employed, for the sake of economy in material and labour. All readings were taken by the direct comparative method (Madsen). Three readings of each series were made; "Standard" agglutination and "trace" at two hours, and "trace" only at 24 hours.

#### SOURCE AND NATURE OF THE SERA TESTED.

Nos. 1 to 20 (inclusive), 26 and 27, were from persons in normal health who had been inoculated with *B. typhosus* during the war. Nos. 2, 3 and 20 had been inoculated with vaccine made in Oxford; No. 14 with American, and the remainder with Army vaccine. Three different "strains" of *B. typhosus* are therefore represented among this number. No. 26 was suffering from Paratyphoid B. fever at the time of bleeding. No. 31 was from a rabbit which had been inoculated with strain T. Lab.; and No. 32 from a rabbit inoculated with strain Edwin; the two last mentioned contained 0.3 per cent. Phenol. The remainder of the sera came from cases of *B. typhosus* infection drawn from N. England, London and Dublin. Two (Nos. 25 and 33) were old stored specimens to which antiseptic had been added in quantities unknown to the writer. The remainder were freshly drawn. Certain of the latter, as will be seen later, were spoiled or infected in the course of preparation and in transmission by post; and some of the few anomalous reactions observed were doubtless due to these accidents.

#### THE SUSPENSIONS USED.

All were standard cultures of *B. typhosus* which had at one time or another been issued from the Standards Laboratory for routine use. Their characteristics are given here in tabular form.

No.	Age	Strain	Agglutinability-factors at time of issue. (Later correction in brackets)		Character of agglutination
T 12	4 yrs	Typhoid Lab.	6.3	—	Good
T 18	3 yrs 2 mths	"	5.8	—	Good
T 19	3 yrs 1 mth	"	6.1	(5.1)	Good
T 21	3 yrs	"	4.7	—	Good. (Rapid clumper)
T 26	2 yrs 6 mths	"	6.7	—	Good
T 32	1 yr 4 mths	Typhoid Edwin	10.3	—	Not good. (Fine clumps; slow to reach maximum; hyper-sensitive)
T 35	1 yr		4.1	—	Good. (Very rapid clumper)



## ARRANGEMENT OF TESTS.

It was intended to test all the sera with a single specimen of each of five cultures, T 18 to T 32; but the frequent opening of the large culture bottles, led eventually to the contamination of some of them. Subsequent sera were tested with three of the same cultures (T 18, T 21, T 32), from a number of smaller bottles, and two more cultures (T 12 and T 35) were added in place of T 19 and T 26 which had been all used up.

The experiments have therefore been arranged in two groups in accordance with the break in procedure.

The preparation and dilution of the sera and cultures, and all the readings, were done by the writer; while the measurements into agglutination-tubes were mostly carried out by Miss J. Jørgensen and Miss E. F. Stubington, both of whom were thoroughly familiar with the necessary technique, and to whom my thanks are due.

A single test only of each serum was made, in order to obtain figures comparable with those obtainable in routine practice.

## CALCULATIONS.

In Tables I, II and III, are presented the relative quantities of each serum required to give the same degree of agglutination with each of the suspensions used.

Each figure was arrived at by taking the middle-term of the three readings, each of which was previously reduced in proportion to the corresponding figure

Table I.

*Proportional quantities of serum required to give equal degrees of agglutination.*

Sera No.	Suspensions					Sera No.	Suspensions				
	T 18	T 19	T 21	T 26	T 32		T 18	T 12	T 21	T 32	T 35
2	72	—	100	102	42	29	75	75	100	78	157
3	67	—	100	129	69	33	86	74	100	40	96
6	76	—	100	118	—	34	74	65	100	—	—
9	62	—	100	96	—	35	70	60	100	35	100
13	67	84	100	83	54	37	77	69	100	55	98
14	66	90	100	94	60	38	75	71	100	47	95
15	74	100	100	100	43	39	69	69	100	40	108
16	67	83	100	94	60	42	78	78	100	49	90
17	58	84	100	105	72	43	85	72	100	40	119
19	65	94	100	83	55	44	73	67	100	46	106
20	67	95	100	102	62	45	67	70	100	49	104
23	70	100	100	75	45	46	75	67	100	65	123
26	79	100	100	79	73	49	75	77	100	56	123
27	64	103	100	78	43	50	53	61	100	37	107
28	72	91	100	63	58	53	68	58	100	40	118
29	74	91	100	65	55	56	68	59	100	41	112
30	76	83	100	77	51	57	100	74	100	—	144
31	71	75	100	70	52	—	—	—	—	—	—
32	65	92	100	80	42	—	—	—	—	—	—
Mean	71.5	91	100	83	50		71.5	69	100	50	107.5

*Agglutination Tests*

Table II. Incomplete Tests.

*Relative serum-quantities.*

No.	T 18	T 12	T 21	T 26	T 32	T 35
7	85	—	100	100	—	—
8	54	—	100	75	50	—
12	61	—	100	122	61	—
36	66	60	100	—	40	100
40	96	85	100	—	58	71

*Ratios of above figures to the means of Table I.*

No.	T 18	T 12	T 21	T 26	T 32	T 35
7	1.19	—	1.00	1.21	—	—
8	0.76	—	1.00	0.90	1.00	—
12	0.85	—	1.00	1.47	1.22	—
36	0.92	0.87	1.00	—	0.80	0.85
40	1.36	1.23	1.00	—	1.16	0.66

## NOTES.

No.

- 7. Incomplete readings.
- 8. Short series.
- 12. Short series. Haemolysis in serum.
- 36. Incomplete readings.
- 40. Short series.

Table III.

*Aberrant figures.*

No.	T 18	T 12	T 21	T 26	T 32	T 35
25	83	—	100	>200	200	81
41	341	318	100	—	545	<90
47	90	83	100	—	71	132
54	120	97	100	—	99	132
55	68	58	100	—	49	164
58	Cannot be stated in figures.					

## NOTES.

No.

- 25. Old 1916 serum, containing unknown quantity of phenol. Slow acid reaction to neutral red. Agglutination very imperfect and peculiar.
- 41. Centrifuge accident. Contamination + +.
- 47. Haemoglobin + + in serum. (Centrifuge accident.)
- 54. T 21 bottle infected; ? others too.
- 55. Haemoglobin + + in serum.

for T 21, which was taken as unity. T 21 was chosen as the basic-standard for the series because it was used in every test. (T 18 would have done equally as well.)

At the base of the columns in Table VII is found the mean of all the observations for each suspension in the whole table.

In the few cases where the short series of tubes was used, the values of the readings have been calculated with Dreyer's reduction table.



In taking T 21 as unity, it is assumed that this culture gave always the exactly true figure. But this is of course not the case, as that suspension is susceptible of experimental and other uncontrolled variations to the same degree as the others. It should be remembered therefore that the actual deviations of T 21 from its mean behaviour with each serum is algebraically summed in each of the figures calculated for the proportional divergence of the other cultures from their mean ratio to T 21.

In other words, each ratio of a suspension to T 21 as unity throws on to the first suspension the whole burden of error, which should in reality be divided between the two. This slight weakness is inherent in all standard methods of comparison in which the standard itself has to be measured, by a method susceptible of some error, each time a test object or substance is to be compared with it.

The figures in Table IV are calculated thus:

The whole column (or columns) of figures in Table I under the heading of each suspension is added up and its arithmetical mean is found (see bottom of Table I). Each value in the columns is divided by the mean for the suspension, and the resulting figures are recorded in Table IV. In theory each should

Table IV.

*Ratios of observations to their arithmetical means.*

Serum	T 18	T 19	T 21	T 26	T 32	Serum	T 18	T 12	T 21	T 32	T 35
2	1.01	—	1.00	1.23	0.84	29a	1.05	1.09	1.00	1.56	1.46
3	0.94	—	1.00	1.56	1.38	33	1.20	1.07	1.00	0.80	0.89
6	1.06	—	1.00	1.42	—	34	1.03	0.94	1.00	—	—
9	0.87	—	1.00	1.16	—	35	0.98	0.87	1.00	0.70	0.93
13	0.94	0.92	1.00	1.00	1.08	37	1.08	1.00	1.00	1.10	0.91
14	0.92	0.99	1.00	1.13	1.20	38	1.05	1.03	1.00	0.97	0.88
15	1.03	1.10	1.00	1.20	0.86	39	0.97	1.00	1.00	0.80	1.00
16	0.94	0.91	1.00	1.13	1.20	42	1.09	1.13	1.00	0.98	0.84
17	0.81	0.92	1.00	1.27	1.44	43	1.19	1.04	1.00	0.80	1.11
18	0.91	1.03	1.00	1.00	1.10	44	1.02	0.97	1.00	0.92	0.99
20	0.94	1.04	1.00	1.23	1.24	45	0.94	1.01	1.00	0.98	0.97
23	0.98	1.10	1.00	0.90	0.90	46	1.05	0.97	1.00	1.30	1.14
26	1.10	1.10	1.00	0.95	1.46	49	1.05	1.12	1.00	1.12	1.14
27	0.89	1.13	1.00	0.94	0.86	50	0.74	0.88	1.00	0.74	0.99
28	1.01	1.00	1.00	0.76	1.16	53	0.95	0.84	1.00	0.80	1.10
29	1.04	1.00	1.00	0.78	1.10	56	0.95	0.85	1.00	0.82	1.04
30	1.06	0.91	1.00	0.93	1.02	57	1.40	1.07	1.00	—	1.34
31	0.99	0.82	1.00	0.84	1.04						
32	0.91	1.01	1.00	0.96	0.84						

be unity. The percentage deviations from theory may be directly read by observing the difference between 100 and each of the figures deprived of its decimal point.

The table may be interpreted thus: if each suspension gave results in every case in accordance with its true degree of agglutinability, all the figures in this table would be 1.00. But a number of unknown or uncontrollable factors

introduce an error into nearly every figure. Each represents the percentage divergence of the actual findings from the theoretical, in comparing each suspension with T 21, which is assumed to yield no error.

For example, since we found in Table I that in general 71·5 parts of serum give as high a degree of agglutination with T 18 as 100 parts do with T 21; this proportion should theoretically be found in every case. But in point of fact there is nearly always a greater or lesser divergence from this ratio.

This being so we must proceed to determine whether these variations are of manageable or of unmanageable proportions; whether, in fact, they throw credit or discredit upon the principle of standardisation, and the use of a reduction-factor.

It will be useful to consider the matter from a second point of view.

In the first place let us consider what is the average and what the maximal deviation from theory in these sets of figures. Analysing them we find a 10 per cent. or greater deviation in 38 per cent. cases; a 25 per cent. or greater in 8·2 per cent. cases; and a 50 per cent. or more (each time actually 56 per cent.) in two cases or 1·2 per cent. In other words nearly two-thirds of observations give less than 10 per cent. deviation; more than nine-tenths give less than 25 per cent., and about ninety-nine hundredths give less than 50 per cent. The mean deviation is about 6 per cent.

It is of considerable interest that the older suspensions T 12, T 18 and T 21 yield much more consistent results than the later ones. Age indeed is all to their advantage as regards stability, but I think that the chief explanation lies in certain imperfections of the war-time materials with which the later suspensions were made. The two most variable are T 26 and T 32, the former made with strain T. Lab. and the latter with strain T. Edwin. The notion of "strain-differences" in the usual sense finds support neither here nor in any other part of these experiments.

Since the agglutinability-factor claims to be an agency whereby any serum can be brought to give (within experimental limits) the same titre with all suspensions, a simple test of its value may now be applied.

If by use of factors calculated from these experiments we obtain a distinctly closer approximation to the ideal of identical titres than is attained without them, the value of the factor is established.

On applying the test to the figures in Table I, we find the results to be overwhelmingly in favour of the factor. In Tables V and VI are set out figures for two pairs of cultures, viz. (a) T 21 with the suspension (T 32) most unlike it in agglutinability, and (b) T 21 with the suspension most similar to it (T 19). The numbers represent the ratios of agglutinability found by comparison of T 32 or T 19 with T 21, the latter always taken as unity.

In the case of the pair T 21, T 32 (Table V) when the figures are unreduced (col. 1), the latter culture yields constantly a very much higher titre than the former, the ratio varying around a mean of 2 to 1.

The second column shows the percentage differences of the "titres" found



for the pair of cultures. *E.g.* the number 2.38 means that the titres were in the proportion of 238 to 100, *i.e.* the former titre exceeds the latter by 138 per cent. The process is then repeated but with the introduction of the reduction-factor, calculated from Table I (T 21 = 1.0, T 32 = 2.0). The figures in the first column are simply divided by the reduction-factor for T 32. In theory the result should be a column of ones; in fact, it is a column of figures varying on either side of one. At the bottom will be found the mean percentage differences, with and without use of the reduction-factor. Without it, the mean is 102 per cent.; with it, only 17.4 per cent. A method whereby titres

Table V.

No. of Serum	Titre found with T 32		Titre found with T 32	
	Titre found with T 21 (No reduction-factor used)	Percentage difference of the two titres	Titre found with T 21 (Reduction-factor used)	Percentage difference of the two titres
2	2.38	138	1.19	19
3	1.45	45	0.73	27
13	1.85	85	0.93	7
14	1.67	67	0.84	16
15	2.33	133	1.17	17
16	1.67	67	0.84	16
17	1.39	39	0.79	21
19	1.82	82	0.91	9
20	1.61	61	0.80	20
23	2.22	122	1.11	11
26	1.37	37	0.69	31
27	2.33	133	1.17	17
28	1.72	72	0.86	14
29	1.82	82	0.91	9
30	1.96	96	0.98	2
31	1.92	92	0.96	4
32	2.38	138	1.19	19
29 <sub>a</sub>	1.28	28	0.64	36
33	2.50	150	1.25	25
35	2.86	186	1.43	43
37	1.82	82	0.91	9
38	2.13	113	1.07	7
39	2.50	150	1.25	25
42	2.04	104	1.02	2
43	2.50	150	1.25	25
44	2.17	117	1.09	9
45	2.04	104	1.02	2
46	1.54	54	0.77	23
49	1.78	78	0.89	11
50	2.70	170	1.35	35
53	2.50	150	1.25	25
56	2.44	144	1.22	22
Mean	2.02	102	1.01	17.4

Reduction-factor improves figures in 31 cases.

Reduction-factor fails to improve figures in one case (see text).

$$\text{Reduction-factor} = \frac{k \text{ of T 32}}{k \text{ of T 21}} = \frac{9.4}{4.7} = 2.0.$$

Table VI.

Serum No.	Titre found with T 19	Percentage difference of the two titres	Titre found with T 19	Percentage difference of the two titres
	Titre found with T 21 (No reduction-factor used)		Titre found with T 21 (Reduction-factor used)	
13	1.19	19	1.08	8
14	1.11	11	1.01	1
15	1.00	0	0.91	9
16	1.20	20	1.10	10
17	1.19	19	1.08	8
19	1.06	6	0.97	3
20	1.05	5	0.95	5
23	1.00	0	0.91	9
26	1.00	0	0.91	9
27	0.97	3	0.88	12
28	1.10	10	1.00	0
29	1.10	10	1.00	0
30	1.20	20	1.10	10
31	1.33	33	1.21	21
32	1.09	9	0.99	1
Mean	1.11	11	1.01	7

Reduction-factor improves figures in 67 % of cases.

Reduction-factor fails to improve figures in 33 % of cases.

are obtained within 17 per cent. of identity is obviously preferable to one which yields a more than 100 per cent. divergence.

In all cases in this table except that of serum 29*a*, there is a closer approach to the ideal identity of titre when the reduction-factor is used, than when it is not; and the gain is usually very great. In the case of serum 29*a* the reduction-factor has actually increased slightly the difference between the two cultures.

That this occurrence is a technical error is made almost certain by the fact that 29*a* is the same serum as 29, under which heading it showed the usual large improvement of ratio on use of the factor.

Table VI shows a similar series of calculations for the pair of suspensions T 21, T 19. Here the agglutinabilities of the two cultures are very close to one another, the relative agglutinability factors being in proportion of 1.0 to 1.1, and therefore no large improvement could be brought about by the use of the factor. Nevertheless, there is a definite improvement; for the divergence from theory is diminished in more than 70 per cent. of instances.

In the remaining 27 per cent. of cases there is an increased divergence, but never amounting to more than 9 per cent.

Tables for the remaining suspensions have not been included, for they all show similar phenomena. The necessary material for calculation is all to be found in Table I, should any reader care to check this statement.

The deductions can be put in another way:

1. If any two cultures differ in agglutinability by 50 per cent. or upwards, the use of a reduction-factor is indispensable for obtaining comparable figures (titres or units) with the two.



2. If they differ by 20 per cent. or thereabouts, the factor will be of limited value for routine tests, though it remains necessary for research in quantitative agglutination-tests.

3. For scientific work a factor is always of value, however much alike in sensitiveness the cultures be.

It may be of interest to record here what were the factors officially given to these various suspensions when they were sent out from the Standards Laboratory and to what degree they differed from the factors calculated from this series of experiments.

It must be explained that the "official" factors are obtained by calculation from a minimum of six full tests of every new suspension against at least two of the preceding standardised suspensions. The sera employed are artificial rabbit or goat sera; and frequently in the past the same serum was used for whole series of tests. Latterly such a procedure has fallen into disrepute, owing to the accumulation of evidence that individual sera may show slight variations in their relative actions on any pair of cultures (Topley, Platts and Imrie, 1919). This is not due to antigenic differences of different "strains" of *B. typhosus* for it occurs when sera and suspensions have all been derived from the same strain. An antigenic variability of the components of any bacterial population is, however, quite a possible explanation and is under investigation at the present time.

The official reduction-factors of our seven suspensions were determined at widely different times, and against different standardised suspensions, and it is therefore putting them to a severe test to make a direct comparison of them all with one another. They are as follows:

Table VII.

Suspensions	Official factors	Factors found in present experiments	Percentage divergence of new, from official factors
T 12	6.3	6.8	7.9
T 18	5.8	6.6	13.8
T 19	6.1	5.2	14.8
T 21	4.7	4.7	0.0
T 26	6.7	5.7	14.9
T 32	10.3	9.4	8.7
T 35	4.1	4.4	7.3
Mean	6.3	6.1	—

This Table VII demonstrates that:

(a) A redetermination of the factors from experiments done with human sera yields results differing in no case by as much as 20 per cent. from the original factors; and on an average by less than 10 per cent.

(b) The mean of the new factors (6.1) differs only by 3.3 per cent. from the mean of the official ones (6.3).

If we proceed to estimate the usefulness of the official factors on the lines

of Tables V and VI, comparing each suspension in turn with T 21, and determining what difference is made by the use of the factor, we find that:

The factor for T 12 improves the results greatly.

„	„	T 18	„	„	„
„	„	T 19	makes the results a little worse.		
„	„	T 26	improves the results slightly.		
„	„	T 32	„	„	enormously.
„	„	T 35	makes no difference.		

Some of these factors were recalculated, and improved, at some interval after they had been fixed for official use (sometimes rather hurriedly under war-pressure). Such was the case with T 19, whose factor was altered (for standardising purposes) to 5.1, a figure which agrees closely with the new factor 5.2.

To the figures given in this paper it will perhaps be objected that a more accurate method of comparison has been employed than that available in routine work, and that therefore a much greater margin of error must be allowed for such work.

No doubt that is true, but in actual fact a pretty close approximation is obtained by use of short-series of tubes, and the reduction-table, when suspensions are compared one with another.

Table II contains the few sera tested in this manner, and it will be seen that, though the average deviations are considerably greater than those in Table IV yet the maximum does not reach 50 per cent.

Since in the curve-tracing of agglutinins for diagnosis (the only "clinical" operation requiring much precision) variations of 100 per cent. are regarded as of dubious significance, and only 200 per cent. or more as of certain value (Dreyer), even a 50 per cent. wobble due to a change of suspension in the middle of the curve will not give rise to errors of judgment.

Nevertheless, as recommended by Topley, Platts and Imrie (1919), it is always well, in such cases, for the worker to do a simple restandardisation test in parallel of the two suspensions with the actual serum in use.

For this will eliminate all chance of confusion.

In conclusion, attention is drawn to Table III, in which I have placed the sera which gave results quite outside the scope of expectation.

The notes appended supply, in my opinion, sufficient proof that the materials were unreliable, and that the figures cannot be included in the main experimental results.

Serum No. 58 was found to give fine traces of flocculation with T 32 (the worst culture of my series), and also with a living saline suspension of *B. typhosus*, up to a dilution of some hundreds. With two other good suspensions it failed to react at all. The most probable explanation of this phenomenon seems to lie in a chemical contamination of the serum, giving rise to a partial clumping of some suspensions; but I could not obtain any actual evidence that this explanation is the true one.



My best thanks are due to the bacteriologists and medical officers who responded so helpfully to my requests for typhoid sera. The work was carried out at Prof. Dreyer's request.

## REFERENCES.

- DREYER, G. (1909). *Journ. of Path and Bact.*, XIII. 331.  
GARDNER, A. D. (1918). *Journ. of Hygiene*, XVII. 471.  
Medical Research Committee (1919). *Special Report Series*, No. 48 (W. W. C. Topley, S. G. Platts and A. C. Imrie).  
SCHROEDER, K. (1909). *Om Aareladningens Indflydelse paa Blodets Agglutinins-Holdighed*. Disputats. Kjöbenhavn.

## THE DISTRIBUTION OF MALARIA IN SOUTH AFRICA AND A MOSQUITO SURVEY OF MILITARY HOS- PITAL AREAS.

BY MAJOR J. PRATT-JOHNSON, M.C., D.A.D.M.S. (PATHOLOGY), S.A.M.C.

THE following information in regard to the distribution of malaria in South Africa has been compiled chiefly from the reports of Government Medical Officers and Health Officials who were circularised by courtesy of the Union Public Health Department in March, 1918, in regard to the following points:

- (1) The areas affected in each district.
- (2) The type or types of malaria existing.
- (3) The incidence of the disease, whether endemic or epidemic; and
- (4) The types of anopheline mosquitoes identified.

The information available from these sources indicates the necessity for a considerable amount of further work to provide a more complete and accurate knowledge of the incidence of malaria in South Africa.

### I. CAPE PROVINCE.

Malaria is practically unknown in the Cape Province. A severe epidemic of malaria occurred in the Northern districts of the Cape Colony, especially along the course of the Orange River in the districts of Gordonia and Kenhardt during 1909, following a period of unusually heavy rainfall. At the same time the disease was reported to be prevalent in Namaqualand and even in the Kalahari, and to a less extent the districts of Vryburg, Mafeking, Taungs, Hay and Barkly West.

This constitutes the only serious recognised epidemic of malaria which has been reported in the Cape Province, cases of Malaria in this Province usually being recorded as being imported from other districts.

Lieut.-Col. Robertson, S.A.M.C., Government Bacteriologist for the Cape Province states that:

The prevalence of malaria in Cape Province is certainly not marked as in my 20 years in Government service I have no record of smears having been submitted from any indigenous case, with the exception of 57 from an outbreak of malaria in the districts of Gordonia, Kenhardt and Upington after extensive flood in 1909. I can find no definite records as to the identification of the mosquitoes in this epidemic and no particulars to show what was the variety of the malarial infection, but as far as I can recollect some of the slides showed Benign Tertian parasites. No information of any kind regarding the types of mosquitoes prevalent in this province is available either in this office or in that of the Government Entomologist.



Captain Ernest Hill, M.C., S.A.M.C. (M.O.H., East London), states that:

In regard to the neighbourhood of East London, I had not prior to my going on military service at the end of 1915 heard of any indigenous cases of malarial fever, and so far as I have been able to learn none have occurred since that time, despite the fact that a good deal of infective material has been introduced. Anophelinae are not common in the municipal area. I have searched for larvae frequently and have found only *Myzorrhynchus paludis*, which I consider to be identical in species with *A. mauritianus*. On one occasion I found a single imago of *Myzorrhynchus natalensis* but did not locate the breeding place.

## II. ORANGE FREE STATE.

The Medical Officer of Health for the Free State (Major Targett-Adams, S.A.M.C.) reports that:

Of 134 medical practitioners approached with a view to obtaining information as to the prevalence of malaria, 36 doctors report that they have met with cases of malaria in their practices, and those who give figures show a *very rough* estimate of some 132 cases; of the 36 medical practitioners who have reported to have seen cases of malaria during their professional work in the Orange Free State, 24 definitely state that the infection was received from places situated *without* the borders of the state, thus leaving 12 doctors who are of the opinion that the source of malarial infection was *within* our boundaries, or that the "fons irigo" was open to doubt. Personally, I am of opinion that if all the factors and their proper correlation do exist for malarial infection to take place within this Province, they are apparently present only to a very slight degree, and are mainly, if not entirely, confined to the immediate vicinity of rivers, such as the Vaal, Vet, Riet and Caledon.

## III. NATAL.

Suitable conditions for the breeding of malarial transmitting anopheles appear to exist throughout a considerable area in Natal, especially the northern areas adjoining Zululand and the coast belt. In Zululand malaria is endemic.

The extensive epidemic of malaria which occurred in Natal in 1905 described by Hill and Haydon (x. 1905, *Journ. Hygiene*, v. 467) drew attention to the presence of suitable conditions in Natal for the spread of malaria. The increased incidence of malaria in Durban in the same year was clearly reflected in the number of cases notified, as malaria had been a compulsory notifiable disease in Durban since 1902.

Year	Cases in Durban	Deaths
1902-1903	47	1
1903-1904	73	1
1904-1905	4314	16

Captain Murison, S.A.M.C., M.O.H. Durban, reported that in February, 1905, malaria became an epidemic disease in Durban. This outbreak of malaria was, in the opinion of the Durban members of the medical profession, the first time, as far as their knowledge goes, that this disease had been of local origin. Had malaria existed in Durban at any time during the past 30 years it could not fail to have been detected.

In a report dated Sept. 1918, Captain Murison, S.A.M.C., states that:

Until the beginning of 1918 no cases of malaria had been reported in Durban for the past ten years.

In December, 1917, a number of Indians returned from East Africa were temporarily accommodated at an Indian Labour Dépôt, in Durban. The camp was situated in a locality in which stagnant water was abundant and anopheline mosquitoes had frequently been found in the area.

Within 50 yards of this Dépôt there are a number of buildings occupied by Indians employed in the Sanitary Department, and within 100 yards a cottage in which two European nurses resided.

In February, 1918, 16 Indians and one nurse contracted malaria.

Six cases also occurred in four houses in the neighbourhood occupied by Europeans and other cases also occurred in the same part of the town.

The parasites discovered in the blood of those examined were of the Benign Tertian type, but not more than 20 per cent. of the cases were so examined prior to the administration of quinine. For the past month no fresh cases have been notified.

The mosquitoes identified from the Indian Camp and its neighbourhood were *Pyretophorus costalis* and *A. mauritanus*.

A mosquito brigade is maintained constantly by the Durban Corporation, special attention being directed to those areas in which malarial transmitting anophelines (commonly *Pyretophorus costalis*) are found from time to time.

#### IV. TRANSVAAL.

In the Transvaal, malaria occurs in the eastern and northern portions and is prevalent in what is known as the low veldt, the high veldt being generally free. Both Benign Tertian and Malignant Subtertian Malaria are found.

In regard to the Eastern Transvaal, Major Spencer, S.A.M.C., of the Middelburg District, reports as follows:

This district is partly highveldt and partly lowveldt, the latter somewhat predominating.

As regards altitude it lies between 4000 feet or somewhat lower in parts of the lowveldt, and 6000 feet in parts of the highveldt. The terms high and low veldt are, however, misleading as representing marked differences in altitude, for an aneroid barometer has shown parts of what is known as the lowveldt to be as high as many parts of the highveldt though each has its special characteristics apart from altitude.

Shortly these characteristics, which are sharply defined, are:

##### *Highveldt:*

More or less Tableland.

Few, small and sparsely scattered streams, with dams, Vleis, and Springs predominating.

Poorly treed and bushed; vegetation sparse.

Subject to fresh breezes which blow for weeks at a time with but slight intermissions.

Climate temperate in summer with frosts of from 10 to 20 degrees for nearly three months of the winter.

##### *Lowveldt:*

Sheltered country falling away from mountain ranges, having the appearance of subsidences and tracts of country washed out by large rivers through the centuries.



Well watered by large and medium-sized rivers, mostly running throughout the year, summer and winter. Well treed, bush often thick and extensive.

Atmosphere more stagnant and evaporation of the earth's surface slow.

Climate almost tropical in summer, mild in winter with few and slight frosts—if any. Vegetation often luxuriant.

The lowveldt is thickly populated by natives, thousands of whom are continually moving up to the highveldt to work, carrying with them malarial parasites with which they are all heavily infected.

Many tours of inspection have been made during the epidemics of malaria which occur periodically in this district during which the numbers found to be affected have been kept and embodied in reports to the Public Health Department. The estimated population of the parts visited has been supplied by the Police in charge of these parts.

During these tours many scores of blood-smears have been taken by myself from those affected and forwarded to the Government Laboratory, Johannesburg, for examination and report.

The estimates and figures quoted are founded upon these investigations and reports.

- (1) Malaria occurs over the highveldt as well as throughout the lowveldt every summer when the rains have set in, but the incidence in the lowveldt is much higher.
- (2) In ordinary seasons Benign Tertian Malaria is endemic throughout the district with an incidence of 10 per cent. of the population of the highveldt and probably 30 per cent. over the lowveldt. In unusually wet seasons Malignant Subtertian Malaria predominates and occurs in epidemic form, with an incidence of from 50 per cent. to 80 per cent. of the population throughout the lowveldt. At such times this infection also occurs over the highveldt with an incidence of 25 per cent. to 30 per cent. It is certain from reports upon the blood-smears taken during these epidemics that to some extent Benign Tertian Malaria is associated with the Malignant infection. Small rings and parasites have invariably been reported in the blood-smears of those affected.

#### NORTHERN TRANSVAAL.

In regard to the Zoutpansberg and Waterberg districts, Dr Robert Kay, District Surgeon, Port Elizabeth, reports as follows:

In 1911 I was appointed M.O. for Northern Transvaal and had the Zoutpansberg and Waterberg districts under my charge. I saw little malaria in either of these two districts during 1911 among the natives—say about 200 cases in all, all of the Benign variety—no deaths. These cases occurred chiefly to the west of Zandrivers Poort—some 40 miles north-west of Nylstroom, and south of Warmbaths. In 1912 there was a considerable outbreak of malaria in Zebedelas Location and along Olifantsriver, along the Zoutpansberg and Middelberg borders. The varieties were of Malignant type and the mortality was considerable. The disease in this outbreak was of an epidemic type—out of say 6000 males, about 200 were fit to walk in Zebedelas Location before treatment, and my arrival on the scene—this sounds rather high, but I do not think it is far short of the facts—malaria was rather rampant all over the Waterberg that year, but no where as bad as the above-mentioned areas. In 1913, as soon as the cold weather commenced, there were further outbreaks in the same areas—but not to any extent so severe as the previous year, the same type—(Malignant)—I found in blood. However, every year malaria—chiefly of Benign type—is to be found along the different rivers in these two districts. As far as I can recollect the anophelines responsible were *Myzomyia*.

## PIETERSBURG DISTRICT (NORTHERN TRANSVAAL).

In reporting on the Pietersburg district (Northern Transvaal), Captain P. A. Green, S.A.M.C., states:

(1) The whole of the Northern Transvaal is liable to outbreaks of malarial infection to a varying extent in different areas, but I have seen no area that has not been infected one year or the other.

(2) Benign Tertian occurs in what is called the Middleveldt, or area above 3000 feet, but also exceptionally Malignant Subtertian. In the Lowveldt, the Benign and Malignant types are about equally prevalent.

(3) There is no question that we get every few years a typical epidemic of Malaria, either spread over a wide area or confined as I have seen it, to one location in a comparatively small area.

SWAZILAND. Malaria is endemic.

BASUTOLAND. Major Macfarlane, S.A.M.C., states:

After 24 years' experience in Basutoland I have only seen a few cases of malaria and these were in natives who had immigrated from Zululand or the Northern Transvaal. I have also seen a few cases in ex-policemen who had been serving in the Bechuanaland Protectorate. My own experience and that of other medical officers of the Basutoland Medical Staff, is that malaria is neither endemic nor epidemic in that territory.

BECHUANALAND. Malaria is endemic along the river beds in the rainy season.

NORTHERN AND SOUTHERN RHODESIA. Malaria endemic and liable to become epidemic during summer rains.

BELGIAN CONGO. Dr Arthur Pearson, who has practised in the Katanga for nearly 20 years, reports as follows:

(1) All parts of the Katanga are affected. There is, however, more malarial infection in the low lying country north of parallel nine degrees than in the mining districts. Infection in the newly sprung up towns and villages has been especially bad hitherto owing to insufficient carrying out of anti-mosquito work.

(2) The type of malaria is invariably Subtertian Malaria. One individual who had been living on the east coast and came to the Katanga for two years, went on holiday to London where he had fever. I then found Benign Tertian parasites. This is the only time I have seen Benign Tertian parasites in any one from the Katanga.

(3) Malaria is endemic. During one rainy season in Elisabethville, it appeared to intensify with epidemic character, but this was due to excessive mosquito breeding in holes dug for ornamental trees, which were not attended to nor filled up.

## A MOSQUITO SURVEY OF MILITARY HOSPITAL AREAS IN SOUTH AFRICA.

In view of the number of sick concentrated in Military Hospitals heavily infected with malarial parasites the question of the presence or absence of malarial transmitting mosquitoes in those areas became a matter of the first importance.

Accordingly arrangements were made at each area laboratory for the collection of local mosquitoes which were subsequently identified by Mr G. A. H. Bedford, F.E.S., whose valuable services became available owing to the kind co-operation of Sir Arnold Theiler, K.C.M.G., Director of the Government



Veterinary Research Laboratory. The majority of the mosquitoes were collected in the larval stage and bred out in the laboratories.

In addition to the material forwarded for identification from military laboratories, specimens were received from Dr Warren, Director of the Natal Museum and Dr Peringuey, Director of the South African Museum.

The following list shows the area in which specimens were collected, the number of specimens identified, and the classification of the different species.

*Classification of species identified.*

Area	No. of specimens	Malaria transmitting	Non-transmitting
Roberts Heights	187	Anopheles costalis	Anopheles squamosus " mauritianus Stegomyia simpsoni Ochlerotatus caballus Theobaldia longiareolata Culex tipuliformis " tigripes " fatigans
Potchefstroom	102	—	Anopheles squamosus B. lineatopennis Ochlerotatus caballus Theobaldia longiareolata Culex fatigans
Cape Town	1542	—	Anopheles cinereus " mauritianus Theobaldia longiareolata Culex tipuliformis " tigripes " pipiens " fatigans " salisburyensis
Durban	286	—	Anopheles mauritianus " squamosus Stegomyia fasciata " simpsoni Culex fatigans
Total ...	2117		

Only two specimens of *Anopheles costalis* were obtained at Roberts Heights. The majority of the specimens from the Cape Town area were *Culex fatigans* and *C. pipiens*. *A. costalis* is also found in certain areas in Durban, but was not found among the mosquitoes collected in the hospital area at Durban.

This paper is included in a report by the Director of Medical Services to the Minister of Defence, Union of South Africa, on "Malarial Research in South Africa during the War."

Acknowledgments are due to Col. P. G. Stock, C.B., C.B.E., Director of Medical Services, Col. G. H. Knapp, D.S.O., Deputy Director of Medical Services, Dr Arnold, M.O.H., Union of South Africa, Lt.-Col. A. Mitchell, Lt.-Col. Haydon, Major Targett-Adams (Assistant Medical Officers of Health, Union of South Africa), Lt.-Col. C. Porter, A.D.M.S., Defence Headquarters, Majors Kenneth Gilchrist, M.C., J. C. Venniker and D. Pullon, and Captains Luke, Impey and Pearson, S.A.M.C., and the Government District Surgeons, without whose ready co-operation it would not have been possible to prepare these records.

# THE SPREAD OF BACTERIAL INFECTION

## SOME CHARACTERISTICS OF LONG-CONTINUED EPIDEMICS.

BY W. W. C. TOPLEY, M.A., M.D. (CANTAB.), F.R.C.P.

*Director of the Institute of Pathology, Charing Cross Hospital.*

*(A Report to the Medical Research Council.)*

(With 5 Charts.)

THE experiments here reported form the preliminary steps in an attempt to investigate the laws which govern the spread of bacterial infection among an animal population, by observations carried out under conditions which are as far as possible experimentally controlled.

For many reasons the mouse has been selected as the most convenient host-species; and the preliminary work has been carried out with *B. Gaertner* as the infecting agent, since a considerable experience has already been gained of infections due to this organism.

In a series of investigations reported elsewhere<sup>(1)</sup>, batches of mice were fed on cultures of *B. Gaertner*<sup>1</sup>. This organism was recovered from those individuals which subsequently died or fell sick, and the cultures so obtained were administered to other batches of mice. This process was repeated again and again, and it was found that certain of the strains isolated showed a greatly increased power of producing a fatal illness, when compared with the cultures earlier employed. After further passage carried out in the same way, this power decreased again, falling to its original level, or even lower.

Certain experiments were then carried out to determine whether the spread of infection by contact showed features which would indicate that such a variation in the character of the parasite is an important factor in the spread of the disease. For this purpose small epidemics<sup>2</sup> were started by placing normal mice in contact with others infected by feeding, and small numbers of mice were subsequently added at irregular intervals to test the risk of developing a fatal infection incurred by entrance to the cage at any given time. The results seemed to indicate that those normal mice added during

<sup>1</sup> This same organism was referred to in the earlier communication as *B. Danyzs*, but since the identity of the bacilli described under these two names seems to be well-established, and the name *B. Gaertner* has the claim of priority, it seems better to employ it.

<sup>2</sup> The word "epidemic" has been preferred to "epizootic" since it is probable that similar causes underlie such processes in whatever kind of living beings they occur, and it seems desirable to emphasise this similarity.



the early stages of such small epidemics had little chance of survival, while of those which were added during the later stages a large part remained alive and apparently well, although at the time of their introduction to the cage deaths were still occurring.

These experiments were not carried beyond this stage. When a sufficient number of deaths had occurred to demonstrate the undoubted spread of infection from the mice originally fed to their healthy fellows, the addition of normal mice was in most cases stopped, and the sequence of events was then studied during the following 30 days or more.

The present investigation has been devoted to observing the effect of continuing the addition of normal mice to an infected cage over a long period of time, and of varying the mode of addition in certain ways.

#### GENERAL TECHNIQUE.

The technique adopted has differed little from that described in the communication referred to above. A certain number of mice have been fed on cultures of *B. Gaertner*. These animals have then been transferred to a suitable cage, and to this cage normal mice have been subsequently added. Each mouse added has been identified so that its subsequent fate could be determined. Each mouse found dead, or killed when dying, has been submitted to post-mortem examination; except in those cases in which the dead mice have been eaten by their companions, or have been too decomposed to render bacteriological examination possible. The more important naked-eye changes have been noted, and cultures have been obtained from the spleen, and from the heart. These have been examined by plating, and testing sub-cultures of selected colonies by agglutination against specific sera; and in many cases by complete fermentation tests. As a routine three colonies from each plate have been examined, when such plates yielded colonies of non-lactose-fermenting bacilli. In many cases more numerous colonies have been tested. It has been found to be of some advantage to submit the primary heart cultures to direct agglutination tests, and this has also been done in the more recent cases.

There are many points of technique not mentioned here, and they have been omitted, not because of their slight importance, but because a really satisfactory method has not yet been worked out. It seems better to reserve this subject for a separate communication, in which the difficulties to be overcome, and the possible ways of dealing with them, may be more fully considered. The experience gained during this investigation has made it clear that a somewhat elaborate technique must be developed and scrupulously adhered to if the spread of infection is to be kept under experimental control. It seems possible indeed that the risk of adventitious infection will always have to be faced, though disturbances caused in this way should not escape notice, and so lead to error.

The question of the health of the normal mice added is, however, so fundamental, that a brief statement must be made of the method adopted to limit

the possible errors due to this cause. Normal mice have been quarantined for at least seven days, usually longer, before introduction to the experimental cage. They have been kept in batches of 6 to 18, and if a death has occurred in any cage, no mouse has been added from that cage; unless all the other mice have remained well for several weeks afterwards, and a complete post-mortem examination of the dead mouse has failed to yield evidence of any known infection. When two or more deaths have occurred in any cage of normal mice the remaining animals in that cage have been destroyed. Among the particulars noted concerning each mouse added to the experimental cage has been the cage from which it came, and the date of transference. To each cage of normal mice is attached a card, stating the source from which the mice were obtained, the number originally in the cage, and the date on which they were brought into the animal house. On this card are recorded any deaths which occur and the post-mortem findings; also the date on which mice have been transferred to the experimental cage. In this way it has been possible to trace the history of any mouse from the time of its arrival, and in great measure to prevent the introduction of sick mice or their immediate contacts into the experimental cage. This procedure has probably sufficed to prevent infected mice being introduced as normal animals without the mistake being subsequently discovered. It has not sufficed to eliminate such adventitious infection. The main experiment under consideration was brought to a close by the appearance in the cage of another unrelated epidemic disease.

#### EXPERIMENT 1.

This experiment lasted from May 21st, 1919, until June 11th, 1920, on which date it was cut short by the appearance in the cage of the infection referred to. The epidemic started about the middle of October, 1919, and continued, in the manner to be described, until the conclusion of the experiment. The more important data are recorded in Chart I. The period, May 21st to September 17th, 1919, is omitted from the chart to economise space. During this time there was no indication of the active spread of infection. 47 mice were added to the cage, 14 of which had been fed on broth cultures of *B. Gaertner* (three separate strains), while 13 deaths occurred, mainly among the mice which had been infected by feeding. The sequence of events during this period is indicated in the table appended to this report. The chart is constructed as follows. On the upper base-line is indicated the number of mice added to the cage on each day. Each unshaded square corresponds to a single mouse. Immediately below this is a curve indicating the total number of mice in the cage. Beneath this again is a base-line on which are indicated the daily deaths, each shaded square corresponding to one mouse. The lowest curve indicates the average survival-time of the mice added to the cage on any given date. In constructing this curve, mice dying in less than three days after introduction to the cage have been disregarded; since, with one exception, no evidence of the infection in question has been found in the small number



of mice dying during the first two days of their sojourn in the cage. The majority of such deaths are apparently caused by fighting. In five cases, where one mouse has survived for a period out of all proportion to the survival-time of its fellows, it has also been disregarded on the ground that it probably possessed a peculiar and individual immunity.

The figures for the survival-times during the second half of May, 1920, and for the 11 days of June, must be regarded as approximate only. At that time a new infection arose in the cage and many of these mice died from this disease. The fresh epidemic arose, however, at a time when an outbreak was to be expected, and the survival-times are probably very nearly the same as those which would have been recorded had the original infection continued its course.

During the 388 days of the experiment, 782 mice were added to the cage and 728 deaths were recorded. On the last day included in this record 46 survivors were present in the cage. There is thus a deficit of eight mice unaccounted for in the chart. One of these was accidentally killed. The remaining seven were recorded as missing on varying dates. The explanation of this is readily found in the tendency of the living mice to eat their dead companions.

#### SUMMARY OF POST-MORTEM FINDINGS AND BACTERIOLOGICAL RESULTS.

It is proposed to publish later a short account of the pathology of this disease as it occurs in mice. It will suffice to point out here, that while very definite changes are often present, yet the bacteriological results form the only trustworthy criterion. In very many cases pure cultures of the organisms concerned may be obtained, while the only change observed post-mortem is a minimal enlargement of the spleen of no diagnostic significance.

Similarly the bacteriological results present many points of interest, which are being further investigated; but it seems better to postpone the discussion of them to another occasion and to include here only those facts which are necessary to demonstrate the nature of the infection studied.

The mice originally infected were fed on a 24 hours' broth culture of *B. Gaertner*. Two other batches of mice were infected by feeding them with cultures of this organism and were subsequently added to the cage during the pre-epidemic period. The strains employed for these latter feedings were isolated from mice which had themselves been fed on the original strain. All these strains were identical as regards their fermentation and serological reactions. Among the mice which died during the earlier stages of the epidemic many yielded pure cultures, not of *B. Gaertner*, but of an organism indistinguishable from it in its fermentation reactions, while failing to agglutinate with a high titre Gaertner serum. Subsequent examination of this organism has placed it in the "Suipestifer" group, using this term in its wide sense. It shows very close serological relationship to several strains of *B. suipestifer* (Mutton). As the epidemic progressed, strains of *B. Gaertner* or of this organism

were obtained from the great majority of dead mice examined. Some mice yielded pure cultures of *B. Gaertner*, others pure cultures of *B. suipestifer*. Very frequently both organisms were obtained from the same mouse. A study of the table appended to this report will show that it is impossible to separate the deaths due to one organism from those due to the other, even could we decide where to place those mice which were doubly infected. The question of the relationship of these organisms is being further studied, but for present purposes the epidemic must be considered as a homogeneous infection. The isolation of *B. suipestifer* from mice experimentally infected with *B. Gaertner* has been reported by many observers, and has been amply confirmed in the epidemic under consideration, in others to be referred to later, and in a series of control feeding experiments.

Briefly, 728 deaths were recorded during the course of the main experiment. Of these, 14 followed direct infection by feeding. Of the 714 mice which were infected by contact, 152 were not examined post-mortem, in most cases because they had been partially or completely eaten by their companions; from 228 mice, cultures of *B. Gaertner* alone were obtained; from 137, *B. suipestifer* alone was isolated; from 130, cultures of both organisms were obtained; while the remaining 67 mice gave negative bacteriological results. In those cases in which *B. Gaertner* or *B. suipestifer* were obtained, they were in almost all instances apparently unmixed with other organisms. The bacteriological results in the case of each mouse are indicated in the table at the end of this report.

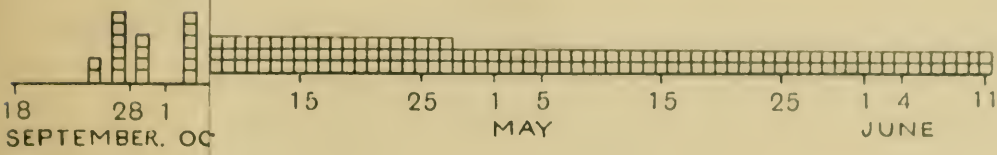
#### GENERAL RESULTS.

The whole experiment may be divided roughly into four periods. The first extends from May 21st, 1919, to September, 17th, 1919, and has been referred to already. From September 18th, 1919, and onwards, only normal mice were added to the cage. From this date until January 5th, 1920, the mice were added in such a way as to keep the total number in the cage roughly constant. Clearly, to add on each day exactly the number of mice found dead might lead to a chance arrangement, in time, of the earlier deaths becoming perpetuated in those occurring later, so that the form of the chart of mortality might come to be only a reflection of the method of addition of normal mice. While this was certainly not the case it is doubtful whether this disturbing factor was altogether avoided. From January 6th to April 27th, 1920, three normal mice were added each day, except on two occasions when none was added. The number of mice in the cage during this period varied from day to day, reaching a maximum immediately before each considerable wave of mortality, and falling to a minimum just before its cessation. During the final period from April 28th to June 11th, 1920, two normal mice were added daily instead of three. In considering the three stages of the actual epidemic it will be most convenient to deal with the last period first, and to join with it the last 25 days of the second epidemic period; for during this time the features



354

NUMBER OF N



TOTAL NUMBER



DEATHS



AVERAGE S



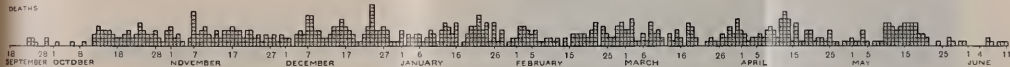
NUMBER OF MICE ADDED.



TOTAL NUMBER OF MICE IN CAGE



DEATHS



AVERAGE SURVIVAL TIME OF EACH BATCH OF MICE ADDED TO CAGE

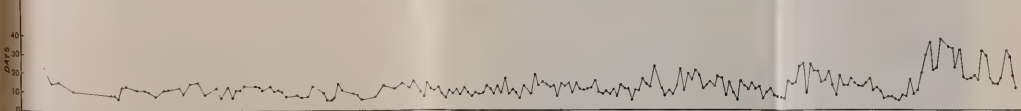


Chart I.



which are of most interest, and which can also be traced in the preceding stages, are more strongly marked and hence more readily studied. The course of events during the period in question, April 3rd to June 3rd, 1920, inclusive, is recorded in Chart II, which is constructed on the same plan as Chart I with certain additions.

A study of this chart shows several striking facts. Although the normal mice were added regularly, the deaths neither occurred with the same

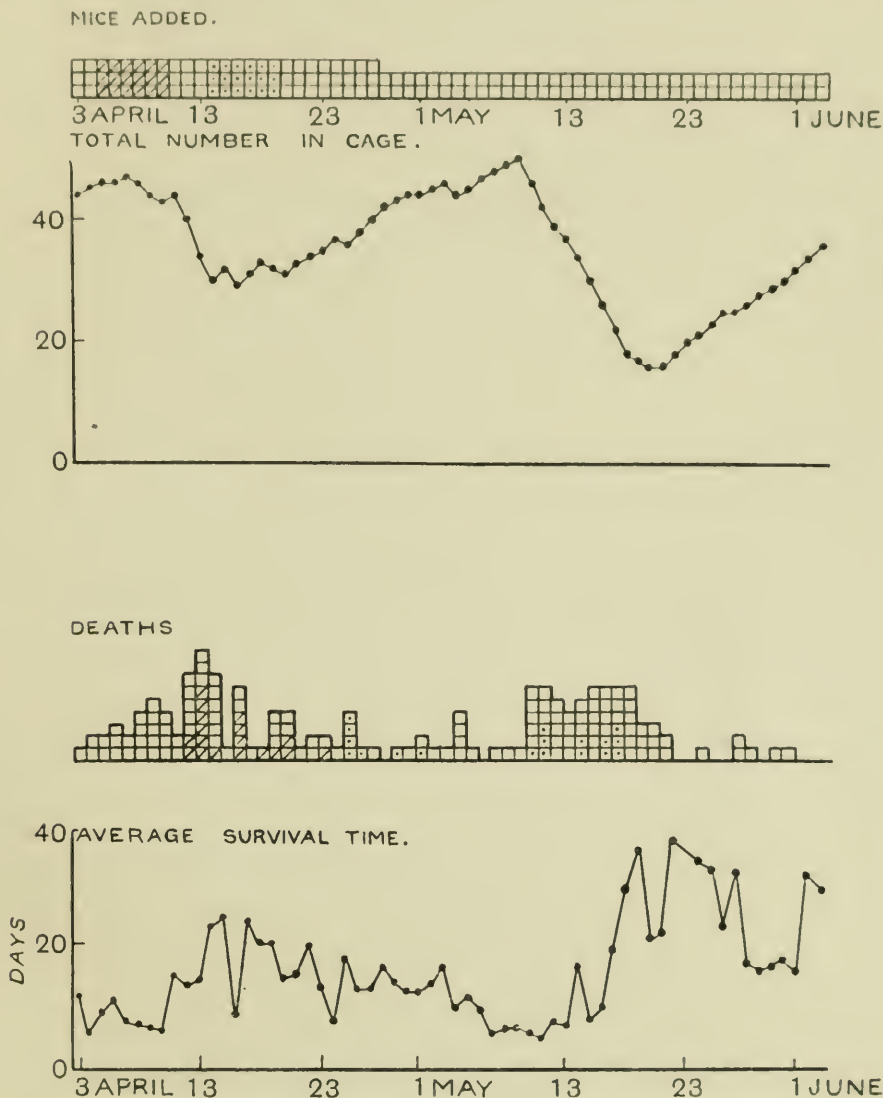


Chart II.

regularity, nor haphazard, but fell into two main groups, within which the daily deaths rose to a maximum and then fell again. Relatively few deaths took place in the intervening period.

If we now compare the distribution of deaths with the curve showing the total mouse population exposed to risk of infection, and with that showing the variation in the average survival-time of the mice added from day to day, certain features present themselves for consideration.

The curve, showing the total cage-population, falls during the latter part

of the rise, and during the crest of the wave of mortality, reaching a minimum at a point slightly preceding the complete subsidence of the wave. It then rises again, reaching a maximum at a point just subsequent to the commencement of a new wave and again passes through the same phases. The curve showing the average survival-time shows corresponding fluctuations, but its maxima correspond to the minima of the cage-population curve, and *vice versa*. The average survival-time of normal mice introduced into the cage is at a minimum during the earlier stages of the rise of the wave, and at a maximum at a point subsequent to its crest but preceding its entire subsidence. Given the fact that the deaths, under such circumstances, occur in waves, it is clear that these results will follow in some measure; but it is worth while to follow a little more closely what actually occurs.

For this purpose two groups of mice have been selected which were added during the first of the two waves. The first group consists of 18 mice, which were added between April 5th and 10th inclusive, during the rise of the wave and when the average survival-time, as indicated by the lower curve, was at or near its minimum. These mice are distinguished among the squares showing the daily additions by marking them with a diagonal line. Their deaths are indicated in the mortality chart by marking the corresponding squares with a similar diagonal. The 18 mice, forming the second group, were added between April 14th and 19th, at a time when the wave was subsiding, and when the survival-time of the added mice was at or near its maximum. The addition and death of these mice are indicated in a similar way to that employed for the first group, a dot being substituted for the diagonal.

Tracing the deaths of these two groups of mice on the mortality chart, we find that all of those added during the rise of the wave died during its crest or subsidence. In this period only four deaths occurred among those mice which were added on later dates. Of the mice added during the second period, none died during this time, although the number of days which elapsed would have sufficed to allow for the death of a considerable proportion of them, judging from the average survival-time estimated for the whole period of the experiment. Seven of these 18 mice succumbed during the inter-epidemic period, but the remaining 11 did not die until the subsequent wave was well under way, and five of the 11 survived until the crest of this wave was passed. Before these 11 mice succumbed, 43 others, added at later dates, had met their death.

It will be well, at this point, carefully to consider the real nature of the facts with which we are dealing. It must be constantly remembered that we are concerned with a curve of mortality and not of morbidity. Were it possible to make an early diagnosis of this type of infection in living mice, our curve would clearly be displaced towards the left. It is hard to say what meaning should be attached to the figure expressing the average survival-time. It is not a measure of the infectivity of the cage-population; for concerning mild infections with a favourable termination our results yield no information.



Nor is it a measure of the risk of death; for this experiment tells us nothing of what the risk of death would be to the mice added at any moment, were the addition of normal mice immediately discontinued. It would seem to indicate, in some degree, the risk of contracting a severe and rapidly fatal infection; but the factors involved are too complex and too uncertain to allow of any descriptive term being employed, which would do other than confuse the points at issue. It seems better to record the survival-times as such, with the reminder that we know little of their meaning.

If now we turn to the earlier stages of the experiment we find the same general features displayed, but less strikingly, because the waves of mortality are no longer distributed in well-marked and relatively isolated groups, so that the end of one wave tends to overlap the commencement of the next. It is clear, however, that during the whole of the period over which the mice were added regularly to the cage, the deaths were neither distributed with the same regularity nor did they occur entirely without order; but showed, though less clearly, a distribution into groups, conforming with varying distinctness to the typical wave-form with its rise, crest and subsidence.

The same features are seen when we go further back to the period during which the normal mice were added in such a way as to keep the total number in the cage relatively constant. Here it is difficult to be certain that the distribution of deaths is not in some degree the reflection of the irregular method of introduction of susceptible mice. The well-marked maxima on December 16th and December 24th may have been largely due to this cause.

The fluctuations in the cage-population curve have already been considered for the period April to June, 1920, and the relation of their maxima and minima to the corresponding points on the mortality curve, and on the curve showing survival-time, has been referred to. If we now trace this curve backwards a very striking feature at once becomes apparent. As far back as the beginning of January, 1920, the same fluctuations are continued, and they show the same periodicity. Taking the whole period January to June there are four and a half such fluctuations. The curve shows five minima and four maxima, the fifth maximal point not being definitely indicated. The period of each complete fluctuation is about 40 days. During the period April to June the two fluctuations in the cage-population curve correspond to two definite waves in the mortality curve. In the period January to April the deaths are more evenly spaced, and do not form two well-marked groups; yet the same periodic fluctuation in the cage-population curve continues, and a closer study of the mortality curve indicates that the smaller groups of deaths, of which it is composed, fall into two large groups, whose maximal and minimal points bear the same relation to the corresponding points on the cage-population curve, as do the better marked maxima and minima of the last two waves of mortality. In the case of the period April to June, it has been seen that minimal cage-population corresponds with maximal survival-time of the normal mice added to the cage. In this respect again, the three curves show

the same relationship during the period January to April, when the curves of mortality and of survival-time are less distinctive. There is thus a strong suggestion that some regularly repeated cycle of events, with a periodicity of about 40 days, underlies the phenomena observed.

A difference in the general form of the cage-population curve may be noted according as the deaths occur in large isolated groups or are more evenly distributed in time. In the former case the epidemic wave causes a rapid decrease in the cage-population, which slowly rises again to its maximum during the period intervening between this wave and the next. The minimal point in such a case occurs not long after the maximum, and the descent towards it is steep, while the ascent to the next maximal point is relatively slow, and the distance, separating the minimum from the maximal point which follows it, is considerably greater than that separating it from the preceding maximum. When the deaths occur in smaller and more evenly distributed groups, the fluctuations in the cage-population curve are less wide, and the minimal points tend to lie approximately midway between the neighbouring maxima.

If we trace this curve still further back, we are unable to follow its fluctuations, since, during the period October, 1919, to January, 1920, the normal mice were added in such a way as to keep the cage-population relatively constant. A very instructive point may, however, be noted. If the process during this period was essentially the same as during the subsequent six months, it might be expected that more mice would have to be added on those dates which would correspond to the minimal points in the cage-population curve during the later stages. An examination of the upper line of the chart, showing the addition of normal mice, indicates that such was the case, though the manner in which mice were added excludes any accurate determination on this point.

The forced termination of this experiment did not allow of any conclusion being arrived at, as to the possible relationship between this periodic fluctuation of the total cage-population and the rate at which normal mice were added, though such seems highly probable. During the earlier stage, when mice were added in such a way as to keep the cage-population relatively constant, the average daily additions were 2.83. During the next period three mice were added daily, except on two days when none was added, the average daily additions for the 113 days being thus 2.95. During the last 45 days of the experiment the daily additions were decreased to two, but the experiment closed before one could expect the appearance of any variation in the periodicity which might have followed this change in the rate of addition. This point therefore has still to be examined.



## FURTHER EXPERIMENTS.

If the results of this experiment, and of those previously reported, be considered together, it is difficult to avoid one very important conclusion. In the present study it has been shown that if normal mice are added from day to day to an infected population they will all eventually succumb, provided that further normal mice are added at a certain rate. The period of survival of any given batch of mice will vary according to the time at which they are introduced to the cage. If their entrance coincides with the early part of the rise of an epidemic wave, as judged by a mortality curve, their survival will be short. If they are introduced during the latter part of the decline of such a wave they will live, on the average, much longer, longer indeed than mice subsequently added to the cage.

In an earlier series of experiments it was found that normal mice, introduced among an infected population during this latter period, seemed in many cases to survive indefinitely, and without showing any observable departure from their normal condition. Should both these findings be confirmed it is clear that, among a population exposed to an epidemic infection, there will usually be a certain proportion who will, if the community concerned be living a relatively isolated existence, survive indefinitely. If, however, fresh susceptibles mingle with this surviving population in any considerable number the epidemic will break out afresh, and among the victims will be those individuals who have passed through the earlier epidemic wave.

There is, however, a very important difference between the earlier experiments and that dealt with in the present report. In the former the addition of normal mice was in all cases stopped after a comparatively small number of deaths had occurred, as the result of contact infection. In the present instance the process has been continued over a long period of time, and hence the virus has been given every opportunity of acquiring its maximal degree of infectivity and of virulence. The two series are therefore not strictly comparable. In order to study more fully the actual effect of the addition of normal mice on the survival of those previously introduced to the cage, two further epidemics were started, by feeding three normal mice in each case with a culture of *B. Gaertner*, and thereafter adding three normal mice daily to each of the two cages. When the mortality curve, combined with the bacteriological results, showed an epidemic of the desired type to be well under way, the additions were stopped and the subsequent course of events observed. In this way conditions were obtained strictly comparable to those existing in the main experiment during the time when three normal mice were added daily.

The course of events is shown in Charts III and IV. The additions, deaths and survival-times are indicated as in Charts I and II. The shaded squares, among those which show the added mice, correspond to animals which survived beyond the time-limits of the experiment. Similarly, the arrow above

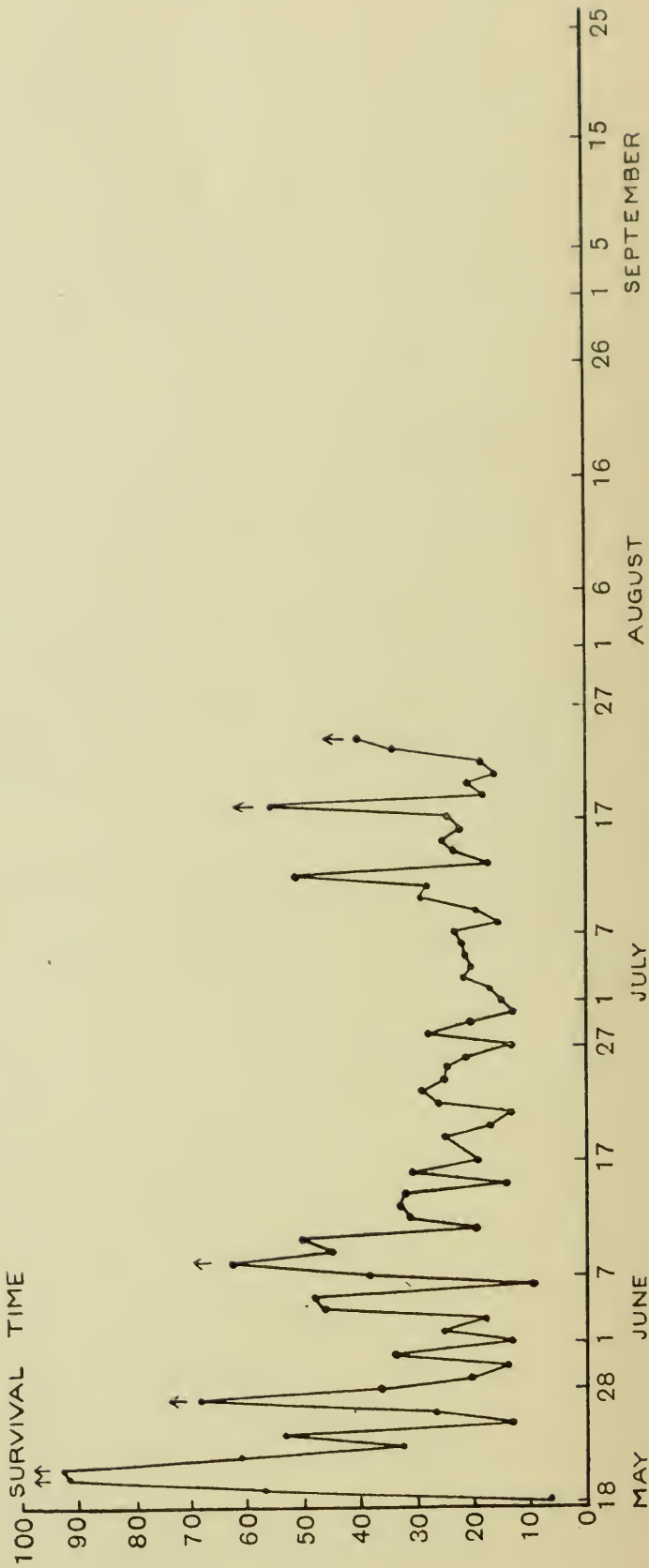
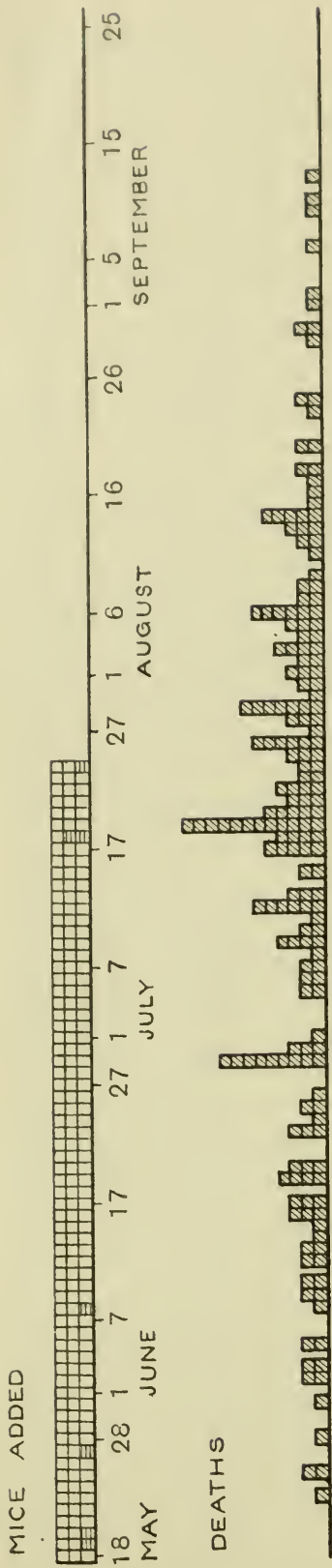
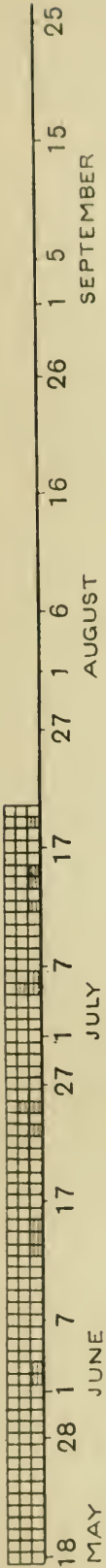


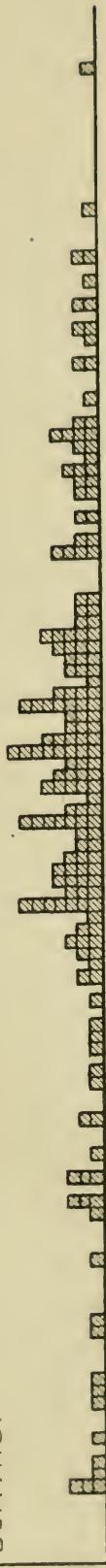
Chart III.



ADDITION OF MICE



DEATHS.



100  
90  
80  
70  
60  
50  
40  
30  
20  
10  
0

SURVIVAL  
TIME

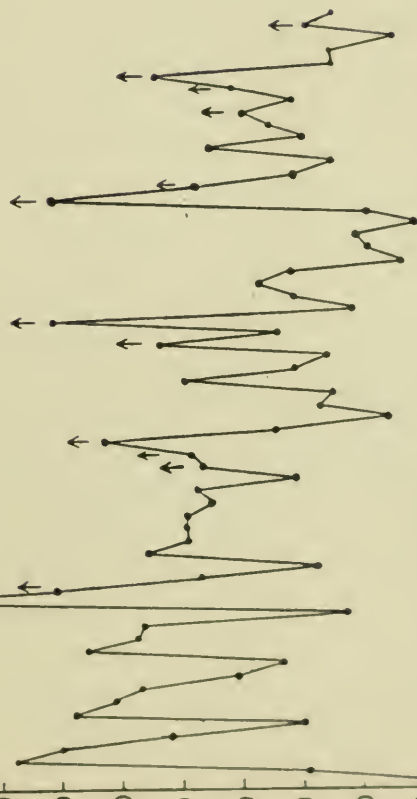


Chart IV.

the corresponding points on the curve of survival-times indicates that the actual average value would have been higher had the experiment been continued longer. Certain points in connection with these charts will be referred to again, but the significant results as regards the effect of stopping the addition of normal mice are set out in Chart V.

In this chart, five dates have been selected during the period of the main experiment when three daily additions were made. They correspond to different phases of the epidemic, and together cover the whole of the period concerned. The deaths of those mice which were alive in the cage on the dates selected have been traced, and the percentage of these mice surviving on each subsequent day is indicated on the chart. Curves A to E have been plotted in this way. Curves F and G indicate, in exactly the same manner, the rate of death of the survivors in the two later experiments after the addition of normal mice had been stopped.

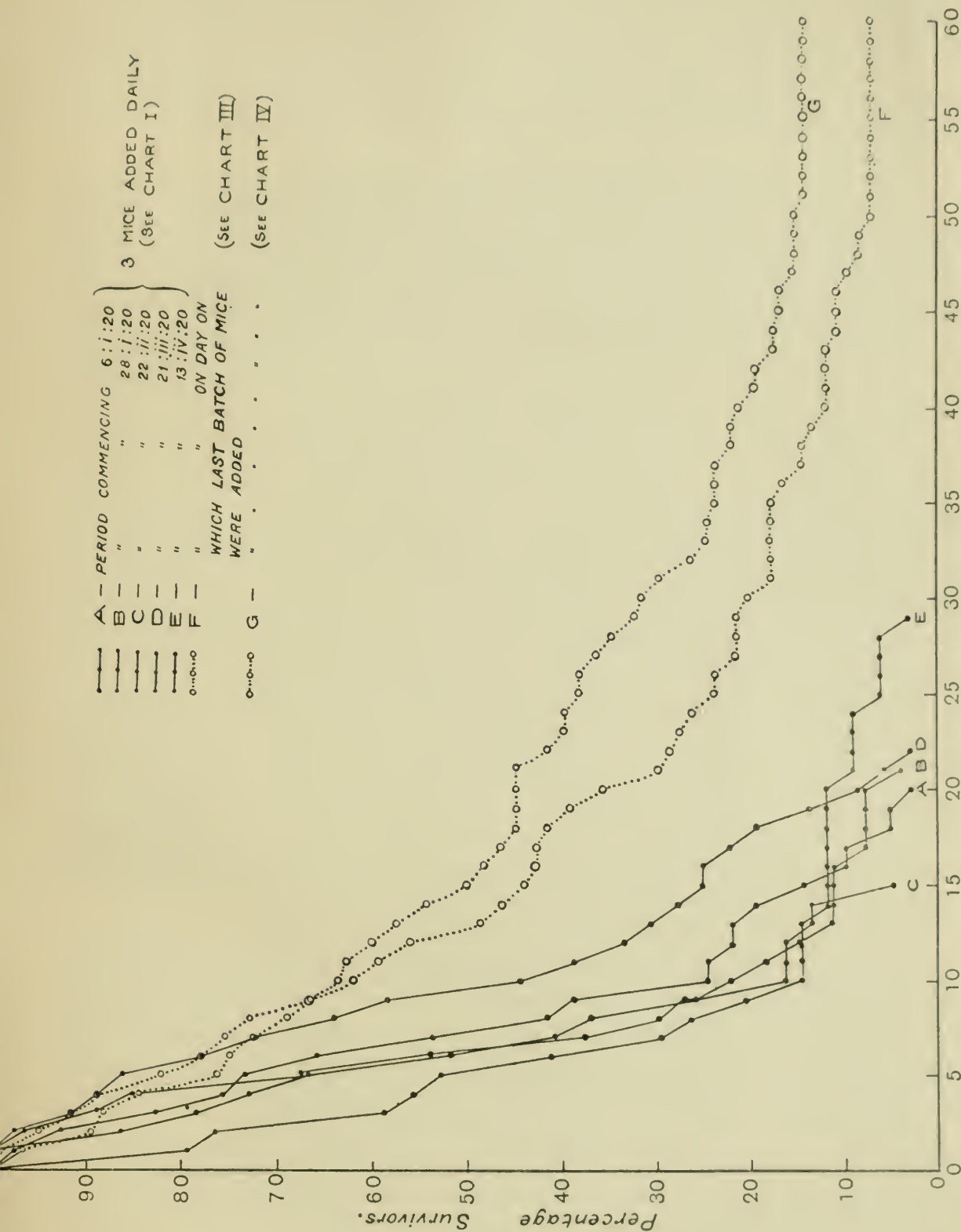
The results are quite definite. Taking the five curves A to E, the survivors had fallen to below 5 per cent. at points varying from the 15th to the 28th day. The actual number of survivors on these dates was two in the case of curve C, and one in each of the others. The fact that the curves do not reach the zero line is due to two mice which survived for a period out of all proportion to the life of their companions.

Taking the two curves, F and G, constructed from the experiments in which no further mice were added after the day on which these observations started, it will be seen that there is nothing distinctive in their course during the first ten days. From thence onward, however, the percentages of survivors in these two cages are markedly in excess of those in the cage to which normal mice were being added. Thus on the 15th day the average figure taken from curves F and G is 47 per cent., from curves A to E 13·4 per cent. On the 30th day, when curves A to E had been discontinued, and when there was in this cage only one survivor, representing less than 5 per cent. of the initial population, 38 per cent. of the mice were still alive in the experiment from which curve G was constructed, and 20 per cent. of those referred to by curve F. On the 60th day the percentages in these cages were 14·5 per cent. and 7 per cent. respectively, and deaths had practically ceased to occur.

It is clear, then, that an epidemic of this kind, which has been allowed to develop to its full tide, even if no further additions be made to the susceptible population, will lead eventually to the death of the great majority of the individuals exposed to risk, but a certain proportion will survive. It is equally clear that the rate of extinction of the surviving population will be very much greater if more susceptible individuals be introduced, and that under these circumstances complete extinction of the original survivors will ultimately result.

One other point must be noted in comparing these results with those obtained in earlier experiments in which the total population exposed to risk was very much smaller. In these cases the survivors under observation were





relatively very few. In the two experiments recorded in Charts III and IV, the surviving population, at the time when the addition of normal mice was stopped, numbered in one case 118 and in the other 84; so that there was ample opportunity for further passage of the parasite to and fro, from host to host. What the result would be of taking a population exposed to the same risk and segregating them in small groups is a problem that has still to be answered.

One other point in connection with these experiments deserves attention. An examination of Charts III and IV shows that, while the mortality curve as a whole rises and falls, yet this large group of deaths is clearly subdivided into smaller groups showing definite maximal points, and that this character is as well marked after the addition of normal mice has ceased as in the earlier stages. The maxima in Chart III occur with a suggestive regularity, and though Chart IV is less regular in this respect they both suggest that the deaths tend to occur in small groups, rising to a maximum and falling again in a period of some five to ten days. Successive groups vary in size in such a way as to form a mortality curve showing a fluctuation with a much longer period. The maximal points in the curve of survival-time, and the distribution among the added mice of those which ultimately survived, suggest as strongly that some fluctuating process with a relatively short period is concerned with the course of events, though here Chart IV is more striking than Chart III. It will be noted too that if the curve of survival-time is to be trusted this fluctuating process is in evidence in the pre-epidemic period. In Chart I, referring to the main experiment, it was noted that the mortality curve showed this same peculiarity, and that, while the cage-population curve showed a fluctuation with a period of some 40 days, the large groups of deaths, corresponding to the phases of this curve, were subdivided into smaller groups. Further reference to this chart shows that these smaller groups exhibit a striking resemblance in their general arrangement and in the interval between their maximal points to those observed in Charts III and IV.

If we try to account for the long survival of certain of the mice, and for the indefinite prolongation of life in some cases, it is hardly possible to believe that the latter have completely escaped infection, or that the former were only infected shortly before the fatal issue. It seems far more probable that these long-lived individuals owed their survival to the fact that, at the time they were added to the cage, the chances were in favour of their acquiring a relatively light infection, which increased their resistance to subsequent attacks of the parasite. It is difficult to account in any other way for the prolonged survival of certain of the mice added during the pre-epidemic period in the experiments recorded in Charts III and IV.



## THE CONDITION OF THE SURVIVORS.

The actual condition of the individuals which remain as the survivors of a considerable epidemic is a point of obvious interest. The epidemic recorded in Chart IV was originally started by feeding three mice upon a culture of *B. Gaertner*, but, from the start, *B. suipestifer* was isolated from the great majority of the mice which died, while *B. Gaertner* was but rarely recovered. On the day when the last batch of normal mice was added to this cage its population numbered 118. 77 days later there were 15 survivors. All of these appeared in perfect health. These 15 mice were killed and examined post-mortem, cultures being obtained from the heart, spleen and liver in each case. The small portion of liver removed included the gall-bladder. The results are given in Table I, together with the more important details in the history of the mice concerned.

Table I.

*Showing P.-M. Findings in Apparently Healthy Survivors from Experiment III (see Chart IV).*

Batch number of mouse	Days in cage	Condition of spleen*	Results of cultures from†		
			Heart	Spleen	Liver
16	125	+	O	S	O
17	124	+	O	S	S‡
27	114	+	O	O	O
28	113	+	O	O	O
29	112	+ -	O	O	O
37	104	+	O	S	S
39	102	+	O	S	S
39	102	++	O	S	S
49	92	-	O	S	S
49	92	+ -	O	S	S
50	91	+ -	O	O	O
56	85	++	S	S+G	O
58	83	++	O	O	O
59	82	+	S	S	O
63	78	+ -	O	O	O

\* - =no enlargement, + - =very slight enlargement, + =moderate enlargement, ++ = great enlargement.

† S=*B. suipestifer*, G=*B. Gaertner*, O =Sterile or lactose-fermenting bacilli only. (In almost all cases the organisms were present in pure culture.)

‡ In this mouse there was a small chronic intraperitoneal abscess just below the anterior margin of the liver which gave a pure culture of *B. suipestifer*.

These results are somewhat surprising. From eight of the 15 mice cultures of *B. suipestifer* were obtained, and from one mouse both this organism and *B. Gaertner*. In the remaining six mice the bacteriological results were negative. In all the positive cases a culture of the organism concerned was obtained from the spleen. In two cases, the heart cultures were positive and the liver cultures negative. In six, the cultures from the liver gave positive results but not those from the heart. In one case *B. suipestifer* was obtained from the spleen alone.

Only four mice showed any marked abnormality post-mortem. In three of these there was marked splenic enlargement. In the other there was a small chronic intraperitoneal abscess, from the pus of which a pure culture of *B. suipestifer* was obtained. The remaining mice, with one exception, showed slight splenic enlargement, but of a degree which is common among mice dying from a variety of causes. With regard to the heart cultures, it should be mentioned that these were obtained by cutting away the apex of the heart and dropping it into a tube of broth. The two positive cultures obtained cannot therefore be regarded as definite evidence of the existence of bacteriæmia.

At this stage there is little point in speculating on the exact meaning of these results. Of 15 apparently healthy mice, 9 were harbouring in their tissues, and especially in the spleen and liver, the causative organism of the epidemic through which they had survived. Their sojourn in the cage had varied between 78 and 125 days. No deaths had occurred in the cage for 14 days and only two during the last month. It seems certain that some state of equilibrium had been arrived at, and that the mice which yielded the positive cultures were acting as carriers. What would have happened had a considerable number of susceptible mice been added at this point? It seems likely that a new spread of infection would have occurred; that a proportion of the newcomers would have died, while the old inhabitants of the cage remained unaffected; but that eventually these in their turn would have succumbed. This point is being more particularly examined in further experiments.

#### SUMMARY AND CONCLUSIONS.

The results so far obtained have raised many questions and answered few of them. The important rôle played in the spread of epidemic disease by the re-accumulation of a susceptible population is clearly indicated. It seems not unreasonable to hope that valuable information as to the effect produced by variations in the rate of such re-accumulation, and on other matters, may be obtained by the satisfying method of direct experiment. The bearing of such information on the well-known fluctuations in the incidence of epidemic diseases, and especially perhaps of those which fall most heavily on children, are too obvious to need emphasising.

The following conclusions seem permissible at the present stage:

(1) If susceptible mice be continuously added to an infected population the spread of infection will continue over a long period of time. There is no evidence that this period has a limit.

(2) When susceptible mice are added continuously and at a constant rate to an infected population, the spread of infection, as judged by a mortality curve, is propagated in regularly recurring waves. These waves are most easily observed by noting the fluctuations in the total cage-population. It seems probable that the period of these fluctuations will be found to depend



on the rate of addition of susceptible individuals, but this point has still to be determined.

(3) The actual deaths may occur in large groups, with intervals during which deaths are few and far between, or they may fall in a succession of smaller groups, increasing and diminishing in size to form the larger waves. In all cases there is this tendency for the occurrence of such small groups of deaths with definite maximal points. There would seem to be two fluctuating processes, the one superimposed upon the other.

(4) The average survival-time of mice added to the cage, and their chance of ultimate survival if no more susceptible mice are introduced, vary according to the phase at which they are added. If they gain entrance to the cage during the rise of a wave they are unlikely to live for long. If they are introduced during the fall of a wave their chances of survival are greatly increased, and they will usually outlive mice which are added at a later date but at a time before the commencement of the next wave.

(5) The rate of extinction of a population, among which infection is actively spreading, will be far less rapid if they are kept isolated, than if further susceptible individuals continuously gain access to them. A proportion of the infected population, which would have survived indefinitely under the former circumstances, will die under the latter.

(6) The ultimate survivors among such a population have not escaped infection, but have successfully resisted it. A considerable proportion of them are harbouring the causative parasite in their tissues.

My sincere thanks are due to my colleagues, Dr H. B. Weir and Dr G. S. Wilson, for their constant help, and to Mrs Phyllis Worthington whose assistance in this work I have been able to obtain by the aid of the Medical Research Council.

#### REFERENCE.

- (1) TOPLEY, W. W. C. (1919). The spread of Bacterial Infection. (Goulstonian Lectures.) *Lancet*, vol. II. p. 1, etc.

## APPENDIX.

Table showing addition and deaths of mice and bacteriological results of post-mortem examinations. (See Chart I.)

G = *B. Gaertner* isolated post-mortem.S = *B. suipestifer* isolated post-mortem.

Nil = Examined post-mortem, but neither organism isolated.

Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
1919					
May 21	6	1	—	—	—
„ 24	6	2	—	—	—
„ 28	6	3	—	—	—
„ 30	—	—	2	1	G
				3	Not examined
„ 31	6	4	1	1	G
June 16	—	—	1	4	Not examined
„ 17	—	—	1	1	G
„ 18	4	5	—	—	—
July 7	6	6	—	—	—
„ 21	—	—	3	6	Nil
				6	G
				6	G
Aug. 19	—	—	1	6	G
„ 26	—	—	1	5	S
„ 30	8	7	—	—	—
Sept. 3	—	—	2	7	S
				5	Nil
„ 15	—	—	1	7	Nil
„ 16	5	8	—	—	—
„ 24	—	—	2	8	Nil
				8	G
„ 25	2	9	2	1	G
				8	Nil
„ 26	—	—	1	8	Not examined
„ 27	6	10	—	—	—
„ 29	4	11	2	10	Nil
				8	Nil
Oct. 2	—	—	1	10	Nil
„ 3	6	12	—	—	—
„ 6	—	—	1	3	Nil
„ 9	—	—	1	11	Nil
„ 12	—	—	3	12	Not examined
				11	S
				10	S
„ 13	8	13	5	12	Not examined
				12	„
				12	S
				3	S
				1	S
„ 14	4	14	4	10	Not examined
				10	S
				9	S
				1	S
„ 15	4	15	4	14	Nil
				7	S
				2	Not examined
				2	S
„ 16	3	16	4	6	G
				2	S
				12	S
				5	G



Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
Oct. 17	5	17	3	11	Nil
				10	S
				12	S
„ 18	—	—	2	15	S
				13	Nil
„ 19	—	—	2	13	S
				3	Nil
„ 20	6	18	3	6	Nil
				13	Nil
				14	S
„ 21	—	—	4	2	G + S
				15	G + S
				14	S
				9	S
„ 22	6	19	2	11	S
				13	S
„ 23	3	20	3	16	G
				13	S
				13	S
„ 24	—	—	6	19	Not examined
				4	„
				13	„
				3	G
				4	S
				17	S
„ 25	8	21	4	3	Not examined
				4	„
				4	S
				14	S
„ 26	—	—	3	15	Not examined
				2	G
				18	S
„ 27	8	22	3	19	Nil
				17	G
				16	S
„ 28	4	23	4	18	G
				17	S
				7	S
				2	S
„ 29	—	—	2	20	Not examined
				21	G + S
„ 30	—	—	1	7	S
„ 31	3	24	4	19	G + S
				18	Not examined
				21	G + S
				21	S
Nov. 1	9	25	5	21	Not examined
				21	G + S
				19	G + S
				17	S
				22	S
„ 2	—	—	2	21	G + S
				18	Not examined
„ 3	5	26	3	15	„
				22	„
				21	G + S
„ 4	—	—	1	18	G
„ 5	2	27	—	—	—
„ 6	—	—	2	21	Not examined
				22	S
„ 7	11	28	9	21	Not examined
				21	G
				21	G
				21	G
				22	Not examined
				22	G + S
				25	S

*Spread of Bacterial Infection*

Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
Nov. 7 <i>cont.</i>				25	S
				17	S
„ 8	—	—	3	23	Nil
				23	Nil
				23	G + S
„ 9	—	—	4	21	G
				19	Not examined
				24	„
				25	G
„ 10	12	29	5	25	Not examined
				25	„
				25	G
				25	G
				22	G + S
„ 11	3	30	3	26	Not examined
				23	G
				22	G + S
„ 12	—	—	2	26	Not examined
				26	„
„ 13	6	31	4	25	„
				25	„
				28	„
				7	„
„ 14	2	32	2	28	„
				28	„
„ 15	2	33	4	28	„
				28	G + S
				31	G
				28	G
„ 16	4	34	4	16	G + S
				29	G
				28	Not examined
				28	S
„ 17	10	35	5	28	Not examined
				28	G + S
				28	G + S
				29	G
				30	G
„ 18	—	—	4	27	G + S
				29	G
				29	S
				30	G + S
„ 19	—	—	4	26	G + S
				29	G
				29	G + S
				30	G
„ 20	7	36	2	29	G + S
				32	G + S
„ 21	4	37	4	29	G + S
				31	G
				31	G
				35	Not examined
„ 22	4	38	4	27	G + S
				28	S
				29	S
				32	G
„ 23	—	—	2	29	G + S
				31	Not examined
„ 24	5	39	3	33	G + S
				31	G
				38	Not examined
„ 25	1	40	1	35	G
„ 26	2	41	3	34	G + S
				35	G
				35	S
„ 27	3	42	2	35	
				37	Nil



Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
Nov. 28	3	43	3	33	G
				34	S
				36	S
„ 29	—	—	1	35	Not examined
„ 30	—	—	3	36	„
				36	„
				38	„
Dec. 1	6	44	2	36	Nil
				38	G
„ 2	2	45	2	26	S
				39	Not examined
„ 4	3	46	3	29	G + S
				37	G + S
				43	G
„ 5	3	47	4	36	G + S
				37	Not examined
				40	G
				43	G
„ 6	—	—	3	39	G + S
				42	G
				44	G
„ 7	—	—	8	35	G
				35	G
				39	G
				39	G + S
				41	Not examined
				41	G
				42	G
				44	G
„ 8	10	48	8	29	G + S
				31	G + S
				35	G
				36	Nil
				38	G
				42	G
				43	G
				44	G
„ 9	13	49	4	35	G
				37	G + S
				45	G
				45	G + S
„ 10	5	50	4	36	G
				44	G
				44	G + S
				46	S
„ 11	2	51	2	46	Not examined
				48	„
„ 12	1	52	2	49	Nil
				49	S
„ 13	4	53	3	39	G + S
				44	G + S
				49	S
„ 14	—	—	5	46	G
				48	S
				49	G + S
				49	S
				49	S
„ 15	—	—	5	47	G
				49	S
				50	G + S
				50	G + S
				50	S
„ 16	17	54	7	47	G
				48	G
				49	G + S
				49	G + S
				49	S

*Spread of Bacterial Infection*

Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
Dec. 16 <i>cont.</i>				49	G + S
				49	G + S
„ 17	5	55	5	48	G + S
				49	G + S
				50	S
				50	G + S
				51	G + S
„ 18	4	56	4	49	Not examined
				51	„
				54	„
				55	Nil
„ 19	—	—	3	48	G + S
				53	Not examined
				54	S
„ 20	—	—	1	48	Not examined
„ 21	—	—	1	54	„
„ 22	9	57	3	48	G
				54	G + S
				56	S
„ 23	—	—	5	48	G + S
				53	G
				54	Not examined
				55	G
				55	S
„ 24	11	58	11	47	G + S
				53	G + S
				54	G + S
				54	G + S
				54	G
				54	G + S
				54	G + S
				54	G + S
				55	G + S
				56	G + S
				56	S
„ 25	—	—	4	54	Not examined
				56	G + S
				57	S
				57	S
„ 26	—	—	4	52	S
				54	G + S
				57	Not examined
				57	S
„ 27	8	59	5	53	G + S
				54	G + S
				54	G + S
				55	G + S
				58	Not examined
„ 28	—	—	4	54	G
				55	S
				58	S
				59	Nil
„ 29	8	60	1	59	Nil
„ 30	—	—	1	58	G
„ 31	6	61	1	57	G + S
1920					
Jan. 1	7	62	4	57	G
				57	G + S
				57	S
				58	G
„ 3	4	63	3	57	G + S
				59	G + S
				62	Not examined
„ 4	4	64	2	58	S
				59	S



Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
Jan. 5	4	65	3	54	G + S
				58	G
				59	Nil
„ 6	3	66	3	58	Not examined
				59	S
				60	G
„ 7	3	67	1	61	Not examined
„ 8	3	68	2	60	G
				64	Not examined
„ 9	3	69	4	60	G
				61	G
				62	Not examined
				62	„
„ 10	3	70	3	61	Nil
				63	Nil
				66	Not examined
„ 11	3	71	1	64	Nil
„ 12	3	72	4	60	G
				61	Nil
				63	Not examined
				67	Nil
13	3	73	6	58	G
				58	G
				59	S
				60	S
				64	Not examined
				69	S
„ 14	3	74	5	58	Not examined
				60	Nil
				60	S
				63	Nil
				64	Nil
„ 15	3	75	3	70	Not examined
				70	„
				63	„
„ 16	3	76	6	59	„
				61	„
				62	„
				65	„
				65	„
				66	S
„ 17	3	77	—	—	—
„ 18	3	78	1	61	G + S
„ 19	3	79	3	68	G
				72	Not examined
				74	S
„ 20	3	80	5	62	G + S
				67	G
				68	S
				69	Not examined
				71	G + S
„ 21	3	81	5	62	G + S
				65	Not examined
				67	S
				69	G
				70	Not examined
„ 22	3	82	8	58	G
				60	G
				68	G
				71	S
				72	S
				72	Not examined
				73	G
				78	G
„ 23	3	83	4	71	G
				77	Not examined
				77	G + S

*Spread of Bacterial Infection*

Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
Jan. 23 <i>cont.</i>				81	Nil
„ 24	3	84	6	62	S
				65	S
				74	G + S
				74	S
				76	S
				79	S
„ 25	3	85	1	76	S
„ 26	3	86	7	66	Not examined
				73	G + S
				73	S
				75	Accidentally killed
				75	Not examined
				76	S
				80	G + S
„ 27	3	87	1	80	G
„ 28	3	88	4	75	Not examined
				77	G + S
				78	G
				79	G
„ 29	3	89	—	—	—
„ 30	3	90	1	79	G
„ 31	3	91	2	83	Not examined
				83	G + S
Feb. 1	—	—	2	82	S
				90	Not examined
„ 2	3	92	5	78	
				82	S
				83	Not examined
				85	
				85	G + S
„ 3	3	93	5	80	G + S
				81	S
				81	S
				87	G
				89	S
„ 4	3	94	4	84	Nil
				87	Not examined
				88	G + S
				89	S
„ 5	3	95	3	82	G
				90	G
				92	Not examined
„ 6	3	96	4	85	S
				87	G + S
				88	G
				90	G + S
„ 7	3	97	1	84	G + S
„ 8	3	98	1	84	S
„ 9	3	99	2	86	G
				91	G
„ 10	3	100	2	86	G
				91	G + S
„ 11	3	101	1	94	G
„ 12	3	102	2	99	Not examined
				100	—
„ 13	3	103	—	—	—
„ 14	3	104	2	88	Not examined
				93	G
„ 15	3	105	—	—	—
„ 16	3	106	3	89	G + S
				92	S
				94	Not examined
„ 17	3	107	3	97	S
				98	G
				106	S
„ 18	3	108	3	86	Not examined



Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
Feb. 18 <i>cont.</i>				96	G
				97	S
„ 19	3	109	3	95	G
				99	G
				99	S
„ 20	3	110	2	95	G + S
				96	G
„ 21	3	111	4	101	G + S
				102	G + S
				103	S
				104	S
„ 22	3	112	6	91	S
				95	S
				96	G
				98	G + S
				103	Not examined
				105	Nil
„ 23	3	113	—	—	—
„ 24	3	114	5	93	S
				94	S
				97	S
				100	S
				103	G + S
„ 25	3	115	3	98	G + S
				101	G + S
				106	G + S
„ 26	3	116	2	101	G
				105	S
„ 27	3	117	2	104	G
				107	G
„ 28	3	118	5	102	G
				106	G + S
				107	G
				110	G
				111	Not examined
„ 29	3	119	6	93	S
				108	G
				108	G
				110	S
				111	G
				112	Nil
Mar. 1	3	120	4	102	S
				105	G + S
				107	G
				115	Nil
„ 2	3	121	4	109	Nil
				113	G
				113	G
				114	G
„ 3	3	122	7	108	G
				110	S
				111	G
				112	G
				113	G
				115	G
				119	Nil
„ 4	3	123	1	119	Not examined
„ 5	3	124	1	115	G
„ 6	3	125	3	112	G
				116	S
				120	G
„ 7	—	—	1	114	G + S
„ 8	3	126	6	104	G
				109	S
				109	G + S
				114	G + S
				117	G + S

*Spread of Bacterial Infection*

Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
Mar. 8 <i>cont.</i>				119	G + S
„ 9	3	127	3	118	G
				118	G + S
				118	G + S
„ 10	3	128	4	116	G
				117	G + S
				120	G
				121	G
„ 11	3	129	2	126	Nil
				117	G + S
„ 12	3	130	—	—	—
„ 13	3	131	3	121	G
				121	G
				127	Not examined
„ 14	3	132	1	129	„
„ 15	3	133	4	123	„
				123	„
				124	G
				128	G
„ 16	3	134	2	116	S
				133	Not examined
„ 17	3	135	—	—	—
„ 18	3	136	6	120	G
				124	G
				124	G
				126	G
				127	G
				135	Not examined
„ 19	3	137	5	129	G
				122	S
				122	S
				127	G
				128	G
„ 20	3	138	2	122	G
				130	Not examined
„ 21	3	139	—	—	—
„ 22	3	140	—	—	—
„ 23	3	141	1	132	G
„ 24	3	142	2	129	Not examined
				132	G
„ 25	3	143	1	130	G
„ 26	3	144	1	128	G
„ 27	3	145	3	123	S
				125	Not examined
				132	S
„ 28	3	146	2	133	G
				134	G
„ 29	3	147	5	125	S
				126	G + S
				130	G
				143	Nil
				145	Not examined
„ 30	3	148	3	136	G
				137	G
				144	G + S
„ 31	3	149	7	131	G
				137	Not examined
				137	G
				138	G
				139	G
				143	G
				146	Not examined
April 1	3	150	4	125	S
				131	G
				140	G
				141	G
„ 2	3	151	3	134	G



Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
April 2 <i>cont.</i>				135	Nil
				146	G
„ 3	3	152	1	138	G
„ 4	3	153	2	138	Not examined
				140	„
„ 5	3	154	2	145	„
				134	G
„ 6	3	155	3	140	G
				145	G
				146	Not examined
„ 7	3	156	2	139	G
				141	G
„ 8	3	157	4	136	G
				143	G
				144	G
				149	G
„ 9	3	158	5	136	G
				139	Not examined
				142	„
				145	G
				153	G
„ 10	3	159	4	34	G
				133	G
				142	G
				153	Not examined
„ 11	3	160	2	131	G
				142	G
„ 12	3	161	7	135	Not examined
				148	„
				151	„
				152	G
				153	G
				154	G
				159	Not examined
„ 13	3	162	9	147	G
				149	G
				151	G
				154	S
				155	G
				156	G
				157	Nil
				157	Not examined
				159	„
„ 14	3	163	7	20	G
				147	G
				147	Not examined
				149	G
				150	Not examined
				152	G
				156	Not examined
„ 15	3	164	1	150	G
„ 16	3	165	6	148	G
				151	G
				155	G
				158	Not examined
				158	G
				158	G
„ 17	3	166	1	150	G
„ 18	3	167	1	156	G
„ 19	3	168	4	100	G
				154	Not examined
				160	G
				162	Not examined
„ 20	3	169	4	152	G
				157	G
				159	G
				160	G

Spread of Bacterial Infection

Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
April 21	3	170	1	161	G
„ 22	3	171	2	141	G
				144	G
„ 23	3	172	2	155	G
				161	G
„ 24	3	173	1	169	Not examined
„ 25	3	174	4	163	G
				165	G
				165	G
				168	Not examined
„ 26	3	175	1	165	G
„ 27	3	176	1	162	G
„ 28	2	177	—	—	—
„ 29	2	178	1	164	Nil
„ 30	2	179	1	173	Not examined
May 1	2	180	2	167	G
				173	Nil
„ 2	2	181	1	174	Not examined
„ 3	2	182	1	172	„
„ 4	2	183	4	161	G
				170	Nil
				170	G
				175	Nil
„ 5	2	184	1	173	G
„ 6	2	185	—	—	—
„ 7	2	186	1	172	G
„ 8	2	187	1	162	G
„ 9	2	188	1	174	Not examined
„ 10	2	189	6	166	„
				169	S
				171	Not examined
				172	Nil
				175	G + S
				180	G
„ 11	2	190	6	166	S
				166	G
				167	G
				171	G
				174	S
				186	Nil
„ 12	2	191	5	160	Not examined
				169	G
				177	G
				178	G
„ 13	2	192	4	179	Nil
				170	G
				183	G
				187	G
				188	G
„ 14	2	193	5	163	G
				167	G
				175	Not examined
				179	Nil
				190	G
„ 15	2	194	6	176	Not examined
				178	„
				181	G + S
				183	G + S
				185	S
„ 16	2	195	6	186	Not examined
				163	„
				164	G + S
				171	Not examined
				184	G + S
				189	S
„ 17	2	196	6	189	Not examined
				164	Nil



Date	Mice added	Batch No.	Mice died	Batch No. of dead mice	Bacteriological results
May 17 <i>cont.</i>				168	S
				168	S
				185	G + S
				187	Not examined
				195	Nil
„ 18	2	197	6	174	Not examined
				176	S
				180	S
				181	Not examined
				184	G + S
				190	G + S
„ 19	2	198	3	177	G + S
				182	G
				188	Nil
„ 20	2	199	3	191	Not examined
				192	G + S
				194	Nil
„ 21	2	200	2	182	S
				192	G + S
„ 22	2	201	—	—	—
„ 23	2	202	—	—	—
„ 24	2	203	1	193	G
„ 25	2	204	—	—	—
„ 26	2	205	—	—	—
„ 27	2	206	2	194	G
				195	G
„ 28	2	207	1	193	Nil
„ 29	2	208	—	—	—
„ 30	2	209	1	199	Nil
„ 31	2	210	1	202	Nil
June 1	2	211	—	—	—
„ 2	2	212	—	—	—
„ 3	2	213	—	—	—
„ 4	2	214	—	—	—
„ 5	2	215	—	—	—
„ 6	2	216	2	196	Nil
				210	Nil
„ 7	2	217	1	200	Nil
„ 8	2	218	—	—	—
„ 9	2	219	1	209	Nil
„ 10	2	220	1	211	Nil
„ 11	2	221	1	198	Nil

# ON ROPE (AND SOURNESS) IN BREAD.

## TOGETHER WITH A METHOD OF ESTIMATING HEAT-RESISTANT SPORES IN FLOUR.

BY D. JORDAN LLOYD, A. B. CLARK AND E. D. McCREA.

(*From the Biochemical Laboratory, Cambridge.*)

### CONTENTS.

	PAGE
1. Introduction . . . . .	380
2. Isolation of causative organisms from bread, flour and grains . . .	382
3. Cultural characters of the organisms . . . . .	383
4. Chemical changes produced in bread by the bacilli . . . . .	386
5. Method of estimation of heat-resistant spores in flour, and distribu- tion of the spores in flour-mills and bakehouses . . . . .	387
6. Conditions controlling the development of rope in bread:	
(i) degree of infection; (ii) moisture; (iii) temperature; (iv) re- action of the bread; (v) composition of the flour . . . . .	390
Summary . . . . .	393
References . . . . .	393

### 1. INTRODUCTION.

DURING the summer of 1917, we were asked by the Food (War) Committee of the Royal Society to investigate the factors responsible for the outbreak of rope in the nation's bread which started early in June and rapidly assumed serious proportions. The following notes form a summary of two reports presented by us to the Committee, and of some further investigations undertaken on behalf of the Wholesale Co-operative Society. We wish to thank both the Royal Society and Dr Geoffrey Martin of the Wholesale Co-operative Society for permission to republish our results.

The earliest reference to the condition now known as rope occurs in a paper by Laurent (1884). He stated that "pain visqueux" was of common occurrence during the summer months amongst the agricultural population of Belgium accustomed to bake their own bread. He ascribed the condition ("pain malade" or "visqueux") to a bacillus which he isolated from the bread and named *B. panificans*. His description of "pain visqueux" is sufficiently striking to be given verbatim: "Pendant les mois les plus chauds de l'année, de juin à septembre, il arrive souvent que le pain préparé dans les ménages de la campagne subisse des transformations d'une nature toute spéciale. Deux ou trois jours après le cuisson il répand une odeur putride; consommé alors, il a un goût sucré qui ne déplaît pas. Peu de temps après, l'odeur devient plus forte, et ne tarde pas à rappeler celle des matières



albuminoides en décomposition. Un couteau introduit dans la mie se couvre d'une matière gluante qui se détache difficilement. Si l'on enfonce le doigt dans la partie centrale de la mie et que l'on le retire lentement, il entraîne des lambeaux qui prennent la forme de filaments analogues à ceux que donne la colle forte."

The cultural characters of Laurent's *B. panificans* are given below. He isolated this organism from ropy bread and also from flour, grains of wheat, and soil. He considered that the organism was universally present on the surface of cereals and passed into the flour during milling. He stated that it could resist the heat of baking, and develop in bread, attacking both gluten and starch. He realised that the activity of the bacillus was dependent on both the moisture and the reaction of the bread, and asserted that "pain visqueux" could be prevented by the addition of vinegar to the dough.

A number of later workers substantially confirm Laurent's conclusions. Kratschmer and Niemilowicz (1889) and Watkin (1906) considered that *B. mesentericus vulgatus* (Flügge) is a causative organism, and Watkin also attributed rope to *B. mes. fuscus*. Uffelman (1890) considered that two types of bacilli called by him *B. liodermos* and *B. mes. vulgatus* (Flügge) could both cause rope in bread. Kries (1893) also attributes rope to a bacillus unspecified. Vogel (1897) made a detailed investigation of rope during an outbreak in Hamburg. He isolated three strains of bacilli of the *Mesentericus* group from affected bread, and considered that two of them, called by him *B. mes. panis viscosus* I and II respectively, were capable of causing rope in bread. Both types produce a highly alkaline reaction in affected bread. Type I is stated to have a proteolytic action on gluten but no action on starch, type II to hydrolise both gluten and starch. Beattie and Lewis (1917) consider that only one species of bacillus (characters not given) can cause rope. They state that the condition of rope is to be attributed to a "viscous fermentation of carbohydrates." They call their organism *B. mes. panis viscosus* (Vogel), but it is to be noted that Vogel states that his commoner type has no action on starch.

All previous workers on rope agree in attributing the condition to a bacillus of the *Mesentericus* group. The cultural characters of the bacilli isolated by them are given in Table I. below. They state that the spores of the bacilli occur on cereals and resist baking. Most workers state that ropy bread is alkaline in reaction and that the condition can be inhibited by the addition of acid to the dough. Several emphasise the importance of moisture and temperature. From their descriptions it is not easy to differentiate between the conditions found in bread in different outbreaks, but it is evident that the bread was not affected in the same way in the various outbreaks which have been investigated. Our own investigations made during the summer of 1917 have led to the isolation from affected bread of five different strains of bacilli all belonging to the *Mesentericus* group, each of which in pure culture is capable of causing the decomposition of bread. These and other bacilli of the *Mesentericus* group can also be isolated from flour, from the outside surface

of grains, wheat, barley, maize, etc., from the dust in mills and bakehouses, and from sound bread. There seems little doubt that they are normally present in all breads, but their presence does not give rise to rope unless conditions prevailing are such as to allow of great multiplication. Under such conditions the changes they produce are:

- (1) an unpleasant smell,
- (2) a brown discolouration,
- (3) a sticky disintegration of the crumb due to the hydrolysis of either the bread proteins or carbohydrates or of both,
- (4) a change in reaction which may become markedly alkaline, markedly acid, or neutral. (Normal unaffected bread has a faintly acid reaction.)

In the alkaline conditions the characteristic stringiness so graphically described by Laurent, develops very rapidly. In the acid or "sour" breads it does not usually occur until decomposition is more advanced. In their early stages of development, it is impossible, except by a chemical and bacteriological examination, to distinguish between the decomposition caused by the different types of infection.

## 2. ISOLATION OF CAUSATIVE ORGANISMS FROM BREAD, FLOUR AND GRAINS.

Affected loaves were examined by the usual aerobic methods, strains of bacteria isolated from the loaves being obtained in pure culture, and re-inoculated into sterile bread to test their action. Five types of bacilli were isolated from affected bread, all belonging to Flügge's *Mesentericus* group. These are referred to as A, B, C, D and E. The loaves were further examined by the methods of anaerobic culture, glucose broth and glucose agar being used. No obligatory anaerobes were isolated by us in either a hydrogen or a carbon dioxide atmosphere. Type A proved to be a facultative anaerobe in both hydrogen and carbon dioxide, types B to E showed slight surface growth in the carbon dioxide atmosphere, none at all in hydrogen. Types A and B were the types encountered most often, C and D were only encountered once each, E three times out of a total of 20 loaves examined.

Whole grains of wheat, barley and maize were examined. Since the only organisms of importance for our investigation were those capable of forming heat-resistant spores, the grains were put into sterile broth or water, and steamed for 20 minutes to destroy non-spore bearing forms. Fifty different samples of grains from every part of the world were examined, every one of which proved to be carrying heat-resistant spores capable of causing unsoundness in bread. Four grain samples from the scourers were also examined and found to be carrying rope bacilli. B was the commonest type, being isolated from 60 per cent. of the grain samples examined; A and E are common; C rather more rare, D was not isolated from any grain sample, but an additional type N was encountered once on a sample of barley. This type N appears to be identical with *B. mesentericus niger* originally isolated by Biel (1896) from bread.



Over 100 samples of flour were examined. 0.5 gram of flour was steamed for 20 minutes in 10 c.c. of sterile water, and the emulsion was inoculated on to agar plates. B, A, E and C are again the types usually found. Type N was encountered twice.

### 3. CULTURAL CHARACTERS OF THE ORGANISMS.

The cultural characters of the strains of *B. mesentericus* A–E and N were examined by growth on various media. Cole and Onslow's tryptic broth was used as the basis for all nutrient media, and the reaction was adjusted to be neutral to phenolsulphone phthalein. ( $P_H = 7.3$  on Sørensen's logarithmic scale (1909)). Gelatine media contained 10 per cent. of gelatine, and agar media 2 per cent. of agar.

A few special media were employed. Bread plates were made by cutting small slices of bread, a quarter of an inch thick, and sterilising them in Petri dishes in the autoclave for 20 minutes at  $115^\circ\text{C}$ . In all experiments with bread plates, control bread plates moistened with sterile water were incubated with the inoculated plates for comparison. Gluten plates were made by mixing gluten flour with tap water in such proportions that a thick, but not sticky, paste resulted. The paste was placed in Petri dishes and autoclaved. During sterilisation the gluten paste "rises" somewhat and becomes of a much firmer consistency. A starch medium was made for testing the action of the bacteria on starch. This was made by adding seven grams of pure cornflour starch to 100 c.c. of neutral broth with phenol red as indicator. The starch suspension was well shaken, tubed, gelatinised by boiling, the tubes being well shaken until gelatinisation was complete, and sterilised in the autoclave. This medium was used for stab cultures. The cultural characters of our types A–E and N are all summarised in Table I, together with the characters of the organisms described by previous workers. The most striking features are the very slight proteolytic power of A, and the very marked proteolytic power of B, C, D, E. It can be seen that the organisms cause a series of decompositions in bread passing from acid to alkaline in the final reactions. Fermentation tests with strains A–E were made with glucose, lactose, saccharose, mannite, and salicin. The results were not specific. The heat-resistant power of the spores of all five organisms is a very important economic character. Pure cultures of types A–E were sealed into five capillary tubes and embedded in dough and baked. The time of baking was three-quarters of an hour. In every case the original organism was subsequently recovered. Vogel's experiments on the influence of the heat of baking had similar results. Experiments with broth cultures at  $100^\circ\text{C}$ . showed that the survival times of the various species differed considerably. Values are given in the table.

Another important cultural character is the inhibition of growth by acid. Growth in all strains is inhibited by a reaction of  $P_H = 5.5$ , a figure which strangely enough coincides with Jesson-Hansen's optimum reaction for gluten. This same limiting value for the growth of rope bacilli was also reached by

Table I.

	<i>B. panificans</i> (Laurent)	<i>B. liodermos</i> (Flügge)	<i>B. mes. vulgatus</i> (Flügge)	<i>B. mes. fuscus</i> (Flügge)	<i>B. mes. panis viscosus</i> I (Vogel)	<i>B. mes. panis viscosus</i> II (Vogel)
Form	...	Bacilli	Bacilli	Bacilli	Bacilli, thick rods	Bacilli, slender rods
Motility	...	—	—	—	—ve	+ve
Gram	...	—	+ve	+ve	+ve	+ve
Spores	...	—	—	—	Central	Position not described
Powers of heat resistance	...	—	—	—	Survives 100° C. for 1 hour	Resists 100° C. for 15 minutes
Anaerobic culture	...	+ve	+ve	+ve	—ve	+ve (slower growth than in air)
Agar slope	...	White rosette-like growth	Grey-white glistening and crumpled growth	—	Grey-brown granular growth, with outgrowths into the medium	Grey-white, dry and crumpled growth
Broth	...	—	Weakly turbid, with a tough membrane	—	Weakly turbid	Clear, with a tough membrane
Potato	...	Gummy, translucent growth	White or pale yellow crumpled growth	Yellow-grey, moist and crumpled growth	White-grey growth, at first slimy, later crumpled	Faintly alkaline
Gelatine stab	...	Funnel liquefaction with membrane formation	Funnel liquefaction, becoming tubular, with membrane formation	Slow funnel liquefaction with membrane formation	Slow funnel liquefaction	White-grey growth, at first slimy, later crumpled
Milk	...	Coagulated, peptonised. Butyric acid produced	Slow coagulation and peptonisation	Coagulated, peptonised. Undergoes a "slimy fermentation"	Precipitation of the casein with slow solution	Rapid funnel liquefaction
Bread	...	Ropy	Ropy	Ropy. Becomes brown, viscous, and evil-smelling	Ropy	Ropy
Gluten	...	—	—	—	Reaction. Alkaline	Reaction. Alkaline
Starch	...	—	—	—	Liquefaction	Liquefaction
Optimum P <sub>H</sub>	...	—	—	—	No change	Rapid liquefaction
Limiting P <sub>H</sub>	...	—	—	—	—	—
Optimum temperature	...	33° C.	—	—	35°–37° C.	40°–42° C.



	Type A	Type B	Type C	Type D	Type E	Type N
Form	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Motility	+ve +	+ve +	+ve +	+ve +	+ve +	+ve +
Gram	Central	Central	Central	Central	Central	Central
Spores	Resists 100° C. for 3 hours	Resists 100° C. for 6 hours	Resists 100° C. for 30 minutes	Resists 100° C. for 6 hours	Resists 100° C. for 1 hour	—
Powers of heat resistance	+ve +	—ve +	—ve +	—ve +	—ve +	—
Anaerobic culture { in H <sub>2</sub> in CO <sub>2</sub>	Grey - white, crumpled and moist growth, with a distinctive bullous appearance closely adherent	Grey - white, moist, opaque and crumpled growth	At first a slimy, white growth, later becoming crumpled	Moist, grey - white growth, punctate appearance, never crumpled (poor growth)	Dry, grey-white, finely crumpled growth	White wrinkled growth, medium stained black
Agar slope	...	...	...	...	...	...
Broth	Turbid, with a tough membrane, bullous in appearance, and of a pinkish colour	At first turbid, later clear, with a tough, crumpled membrane	Turbid, with a thin membrane	Slowly becomes turbid, with a slow formation of a thin film	Slight turbidity, with a tough, crumpled membrane	—
Potato	Alkaline	White-yellow, crumpled growth	Slimy white growth, later crumpled	White crumpled growth	Green - yellow, finely crumpled growth	—
Gelatine stab	Pink - brown, finely plicated growth, bullous appearance, adherent	White-yellow, crumpled growth	...	...	...	...
Milk	Extremely slow liquefaction	Rapid funnel liquefaction	Liquefaction	Slow liquefaction	Liquefaction	—
Bread	Coagulated	Precipitation of the casein with peptonisation	Precipitation of the casein with peptonisation	Precipitation of the casein with peptonisation	Precipitation of the casein with peptonisation	—
Gluten	Acid	Alkaline	Alkaline	Alkaline	Alkaline	Inky stain, softened
Starch	Pinkish - brown stain, softened, sticky and crumbly	Yellow to dark brown stain, consistency resembling that of honey	Green - yellow stain, softened, sticky and crumbly	Yellow - brown stain, consistency as B	Green - yellow stain, softened, sticky and crumbly	Acid
Neutal	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	—
Surface growth. Not noticeably softened	Surface growth. Not noticeably softened	Surface growth. Gluten becomes a slimy, semi-liquid mass	Surface growth. Gluten much softened	Surface growth. Gluten becomes slimy and semi-liquid	Surface growth. Gluten much softened	—
NH <sub>3</sub> given off. Alkaline	NH <sub>3</sub> given off. Alkaline	NH <sub>3</sub> given off. Alkaline	NH <sub>3</sub> given off. Alkaline	NH <sub>3</sub> given off. Alkaline	NH <sub>3</sub> given off. Alkaline	—
Slow liquefaction. Pellicle formed	Slow liquefaction. Pellicle formed	Rapid liquefaction	Rapid liquefaction	Rapid liquefaction	Liquefaction	—
Alkaline, then acid	Alkaline, then acid	Acid	Alkaline	Acid	Reaction unchanged.	—
Optimum P <sub>H</sub>	6.5	7.5	6.5	7.0	P <sub>H</sub> 7.4	6.5
Limiting P <sub>H</sub>	5.5	5.5	5.5	6.0	...	5.5
Optimum temperature	About 37° C.	About 37° C.	About 37° C.	About 37° C.	About 37° C.	About 37° C.

Cohn in some subsequent work on rope in America. The work is reported by Henderson (1918).

#### 4. CHEMICAL CHANGES PRODUCED IN BREAD BY THE BACILLI.

These fall readily under three headings:

- (1) Changes in reaction.
- (2) Changes affecting the proteins.
- (3) Changes affecting the carbohydrates.

Changes in reaction were tested by inoculating sterile bread plates with pure cultures, incubating for 48 hours at 37° C. and testing with indicators, and comparing the colour with that of a sterile control bread plate. The error is about  $\pm 0.25$  on the scale of units used. The results are given below.

Table II. 48-hour bread cultures.

Bacillus	Consistency of bread plate	Colour of bread plate	Reaction of bread plate	Biuret test on watery extract
Control	Normal	Normal	Neutral to brom-cresol purple. $P_H = 6.5$	Negative
A	Sticky, softened	Pink, later brown	Acid to phenol red, alkaline to brom-cresol purple. $P_H = 7.0$	Negative
B	Very viscous, semi-liquid	Brown	Alkaline to phenol red and litmus. Neutral to cresol red. $P_H = 8.0$	Pink colour
C	Sticky, softened	Greenish-yellow, later brown	Acid to phenol red, litmus, brom-cresol purple. Neutral to methyl red. $P_H = 5.5$	Mauve colour
D	Very viscous, semi-liquid	Yellow, later brown	Alkaline to all indicators used. $P_H = 9.0$ (circa)	Pink colour
E	Sticky, softened	Yellow, later brown	Acid to litmus, phenol red and brom-cresol purple. $P_H = 5.5$	Mauve colour

There seems to be a close relation between the reaction produced by the infecting bacillus and the destruction of the bread proteins. Type A which produces a very slight shift of the reaction towards the alkaline side, has very little proteolytic power—too little to detect in a 48-hour bread culture; grown on gluten, the gluten plate becomes covered with a pink encrusted growth, there is a smell of ammonia, showing that the protein is de-aminated but the gluten remains firm and consistent, and no water soluble bodies are produced that give the Biuret reaction. B and D which produce alkali on bread and on gluten, rapidly liquefy both media. The advanced stage of proteolysis is shown by applying the Biuret test to watery extracts of bread and of gluten. In both cases a deep rose-pink colour is produced. In bread infected with B, it is readily shown by a formol titration, that not only albumoses and peptones, but also large quantities of free amino-acids are produced.

A and C which produce an acid reaction in bread, have a less violent proteolytic action, possibly because the acid produced is sufficient to bring the reaction near to a value which inhibits growth.



Laurent and Vogel both find that protein break-down occurs in breads infected with the bacilli isolated by them.

Cultures of the bacilli on a gluten medium, however, show a series of protein liquefactions exactly parallel to the series on bread, though in every case on gluten the medium becomes alkaline and there is evolution of free ammonia.

This difference of reaction produced on bread and on gluten by the five types under consideration is of considerable chemical interest. The reaction in the bread cannot be explained simply as the sum of the reactions of the various organisms on the protein and carbohydrate respectively, since though A, B, D and E all produce an acid reaction in starch stabs, C, which is an acid producer on breads, produces alkali not only from gluten but also from starch.

Normal bread contains starch, dextrins and reducing sugar. Laurent states that his *B. panificans* attacks cooked starch with the production of an erythrodextrin. Vogel stated that *B. mes. panis viscosus* II had a strong diastatic action and that in an infected loaf there was an accumulation of reducing sugars, mainly maltose and glucose. We have found that in bread infected with our type B there is an increase of a reducing sugar identified as glucose. The quantity of reducing sugar in the loaf may be used as an index of the progress of the decomposition. Hüppe (1884) has shown that *B. mesentericus* as a group is characterised by possessing a diastatic enzyme and Effront (1917) considers that this enzyme is an aerodextrinase.

#### 5. METHOD OF ESTIMATION OF HEAT-RESISTANT SPORES IN FLOUR, AND DISTRIBUTION OF THE SPORES IN FLOUR-MILLS AND BAKEHOUSES.

The method described below for estimating the degree of infection of flour with deleterious bacteria has been published in the reports of the Food (War) Committee of the Royal Society (Jordan Lloyd and McCrea, 1917). The figures for infection of flours given in this report were all obtained under war conditions, but we feel that it would be of more general value to give here results obtained under a more normal state of affairs. We have, further, standardised our method of examining flour; a detailed description is given below:

(1) 300 c.c. of distilled water is placed in a 500 c.c. flask. This is plugged with cotton wool and autoclaved.

(2) 100 g. of the flour is weighed out and shaken into the sterile water with constant stirring.

(3) The flask containing the flour and water is well shaken.

(4) Tubes containing 10–20 c.c. of nutrient agar are melted, four of these being allowed to each sample of flour.

(5) 4 c.c. of the flour emulsion is removed from the flask with a sterile graduated pipette, 1 c.c. being placed in each of the four tubes of melted agar.

(6) The tubes are placed in boiling water for 20 minutes.

(7) The contents of each inoculated tube are poured into a sterile Petri dish.

The colonies of *Bacillus mesentericus* are counted after 24 hours' incubation at 37° C.

The flour is then judged by the total number of colonies on the four dishes. If there are 12 (or less than 12) colonies, the flour is to be considered sound.

If there are between 12 and 50 colonies, the flour is liable to develop rope under bad conditions.

If there are more than 50 colonies, the flour is to be regarded as unsafe for ordinary commercial use.

Experimental results are given below. They show the low degree of infection in normal flour, the order of infection found where rope has occurred, and the stages in the milling process at which the rope spores are liable to accumulate.

Case 1. *Samples collected from a flour-mill.*

Number of sample	Description	Total number of colonies on the four plates	Dominant type of <i>B. mesentericus</i> (1)
1	Wheat berry ground up	14	A, B and C
2	Break flour	22	A, B and C
3	"Dead" flour from break rollers	∞	A, B, C, E, etc.
4	"Dead" flour from ledges and "sweat" from break middling machine	∞	A, B, C (and maggots!)
5	Crude semolina	2	—
6	Pure semolina	1	—
7	"Dead" flour from ledges of semolina machine	17	A and C
8	Finished flour from sack	7	A
9	Finished flour from different sack	5	A
10	Bran	28	A, B and C

Case 2. *A further series of samples from the same flour-mill as Case 1.*

Number of sample	Description	Total number of colonies on the four plates
1	Manitoba wheat from silo S. 9 ... ..	2
2	Manitoba wheat going on drier from whizzer ...	0
3	Manitoba wheat leaving drier ... ..	3
4	River Plate wheat from silo S. 10 ... ..	8
5	Plate wheat going on drier from whizzer ...	0
6	Plate wheat leaving drier ... ..	0
7	English wheat from finch ... ..	2
8	English wheat going on drier from whizzer ...	2
9	English wheat leaving drier ... ..	5
10	Australian wheat going on drier from whizzer ...	1
11	Australian wheat leaving drier ... ..	4
12	American winter wheat from silo M. 3 ... ..	8
13	Clean winter wheat after standing 12 hours ...	4
14	American maize nuts ... ..	36
15	Aspirations from wheat separator ... ..	16
16	Mixed wheat going on mill after standing 12 hours	4
17	1st break ... ..	2
18	2nd break ... ..	2
19	"Dead" flour from second break rollers ...	4
20	Reduction semolina A ... ..	1
21	Reduction semolina B ... ..	12
22	Reduction semolina C ... ..	4
23	Finished flour ... ..	18



Case 3. *Samples collected from a different flour-mill.*

Number of sample	Description	Total number of colonies on the four plates
1	Winter wheat going on drier from whizzer	0
2	Winter wheat leaving drier ... ..	0
3	Grind 1st break ... ..	3
4	Grind 2nd break ... ..	3
5	Break flour ... ..	1
6	Fine middlings ... ..	2
7	Semolina ... ..	0
8	Finished flour ... ..	0

Case 4. *Samples of flour taken from a bakery where rope had occurred in the bread.*

Number of sample	Description	Total number of colonies on the four plates	Dominant type of <i>B. mesentericus</i>
1	Flour scrapings from floor and ledges in flour stores	610	A 30 % B 60 %
2	Flour in bag attached to automatic mixer	130	A 50 % (and numerous B 50 % small beetles)
3	Flour adhering to canvas shoot from hopper	30	A 50 % B 50 %
4	Brushings from outside ledges of a trough	2000 (about)	A 30 % B 30 % <i>B. subtilis</i> 30 %
5	Flour I	342	A
6	Flour II	1	—
7	Flour III	5	—
8	Flour IV	5	—
9	Flour V	3	—
10	Flour VI	4	—
11	Flour VII	7	—
12	Flour VIII	7	—

In case 4, out of eight flours examined, seven were "sound" and one was found to be heavily infected with *B. mesentericus* type A. The bread, however, on examination showed that the rope was the alkaline type characteristic of *B. mesentericus* type B. The source of infection, therefore, was not primarily to be traced to flour I but to the dirty condition of the bakery, as shown by samples 1-4.

Case 5. *A second series of samples from the same bakery as case 4.*

Number of sample	Description	Total number of colonies on the four plates	Dominant type of <i>B. mesentericus</i>
1	Flour scrapings from ledges in flour store	800	A, B and C, and numerous others not identified
2	Brushings from the outside ledges of a trough	145	As above
3	Dough from flour of mixing room	1000	A, B, E, etc.
4	Flour I (sample milled a week later than in case 2)	114	A
5	Flour I (sample milled 3 days later still)	11	A

These examples show very clearly how an infected mill will clean itself out during subsequent millings, the three samples of flour I showing degrees of infection 342, 114 and 11, all being milled within ten days of each other.

#### 6. CONDITIONS CONTROLLING THE DEVELOPMENT OF ROPE.

We have shown that spores of the *Mesentericus* group are universally present in flour and bread, but their presence is a matter of no importance unless conditions are such as to favour great development. The five principal factors concerned in such development are:

- (i) Degree of infection.
- (ii) Moisture.
- (iii) Temperature.
- (iv) Reaction of the bread.
- (v) Composition of the flour.

(i) *Degree of infection.* Watkin (1906) and Semler (1917) give instances of occurrence of rope attributable to highly infected batches of flour; Beattie and Lewis give an instance where indirect evidence suggested that an increased degree of infection was the cause of an outbreak. We have had the opportunity of examining the flour connected with a serious outbreak of rope in a military camp, and found it very highly infected. In another case where rope developed in two loaves from a certain baker's shop, the flour was not infected to a greater degree than the normal war-flour of the year 1917. The bakery is a very reputable one, and both loaves came from a batch of bread baked on a Friday and kept over a week-end during a spell of very hot weather in June. In the instance of rope in a bakery analysed in a previous section, it was shown that though an infected flour was concerned in the outbreak, the main source of trouble was the contamination of the dough in the bakehouse. This case is a good example of how infection in bread may come from both internal and external sources.

(ii) *Moisture.* The importance of the moisture content of a loaf was stated by Vogel and is indeed clearly recognised in the practice of modern large-scale bakeries where the freshly drawn bread is placed on the floor of the store house which is arranged with a through draft to draw off the steam rising from the warm loaves. In the early days of the enforcement of the 24 hour bread order, the freshly baked bread was frequently stacked while hot and steaming in the delivery carts, or some other closed space in order to prevent loss of water and to maintain the weight of the loaf. This habit as may be imagined led to a considerable number of cases of rope. The following experiment shows the influence of the moisture content of the loaf on its capacity to develop rope. A dough was made in the usual manner and divided into portions each weighing one pound. After proving, the doughs were all baked together in a slow oven at 125° C. They were withdrawn at different times. Loaf III may be considered a "normal" loaf, loaves I and II were under-



baked, loaves V and VI over-baked. After baking and cooling the loaves were cut in two, and the control halves were estimated for reducing sugars. The other halves were incubated for 48 hours in a moist incubator at 37° C. All developed rope of the B type. The reducing sugars were then estimated in the ropy halves. In all loaves the method of estimation was the same. Fifty grams of bread from the middle of the loaf were extracted three or four times with boiling water. The total volume of the extracts was made up to 250 c.c. Each extract was mixed with 500 c.c. of alcohol to precipitate the dextrins, allowed to stand 24 hours and filtered. The alcohol was removed by distillation *in vacuo* and the total again made up to a known volume. 125 c.c. was found to be a convenient volume for extracts from unincubated, and 250 c.c. for extracts from incubated loaves. The results are given in the table below. The sugar was estimated by Benedict's method. The amount of sugar present is expressed as a percentage of the weight of the uncooked dough.

Table III.

Loaf	Time of baking, mins.	Weight, oz.	Sugar in control half, %	Sugar in ropy loaf, %
I	30	15.5	1.1	6.38
II	45	14.5	1.0	11.8
III	60	13.75	.9	9.5
IV	75	12.9	1.0	7.23
V	90	12.25	.9	4.73

This experiment illustrates two points: (1) that *B. mesentericus* does not readily attack uncooked starch, (2) the importance of the moisture content.

(iii) *Temperature*. Rope is essentially a warm weather trouble as is recorded by Laurent, Vogel, Watkin, etc. The outbreak with which we were concerned was at its height in June, but cases continued to occur throughout the summer.

(iv) *Reaction*. The reaction of the growth medium is well recognised as having considerable influence on the rate of growth of micro-organisms, and acid breads have been found by all investigators of rope to be immune from the condition. An experiment was made by adjusting nutrient broths to varying reactions, to investigate the limits of growth of *B. mesentericus* types A-E. The broths were adjusted according to the instructions in Cole and Onslow's paper (1916). The results are given in the table below. 0 signifies no growth; + very slight growth; ++ slight growth; +++ good growth; ++++ very heavy growth. The results are taken from 36-hour cultures.

Table IV.

Reaction of broth	<i>Bacillus mesentericus</i> type				
	A	B	C	D	E
P <sub>H</sub> = 8.0	(+)	(+)	(+)	(+)	(+)
7.5	+	++	+	++	+
7.0	++	+++	++	+++	++
6.5	+++	+++	+++	++	+++
6.0	++	++	++	0	++
5.5	0	0	0	0	0
5.0	0	0	0	0	0

It can be seen that the optimum reaction for growth is  $P_H = 6.5$ . This unfortunately coincides with the reaction of normal bread. The inhibitory reaction  $P_H = 5.5$  coincides with Jesson-Hansen's value for the reaction to be desired in a dough in order to attain the optimum condition of the gluten. It is, therefore, from both points of view the theoretical ideal for bread. Its attainment in bakehouse practice, however, presents difficulties.

(v) *Composition of the flour.* There is a very definite belief in the baking trade that brown bread is more liable to rope than white bread, and much circumstantial evidence tending to confirm this. This difference might be due to a varying in the degree of infection, the natural supposition being that brown flour containing branny particles would have a larger number of bacteria than white flour from the middle of the wheat berry, or it might be due to the brown bread being a more stimulating medium of growth. Both these possibilities were examined. Only one sample of wholemeal flour was available at the time the examinations were made. It certainly had a high degree of infection, 180 colonies developing on the agar plate compared with values varying from 30 to 70 obtained from flour of 70, 80 and 90 % extraction. The differences among the latter were not sufficiently striking to have been due to anything but chance. The possible influence of the different lengths of extraction on growth was tested in the following manner: 100 grams of flour were added to flasks containing 250 c.c. of sterile water and the reaction was adjusted to be neutral to phenol red. The contents of the flasks were digested for four hours at  $37^\circ \text{C}$ ., and then centrifuged. Thirty cubic centimetres of the watery extract were drawn off and sterilised by the intermittent method at  $60^\circ \text{C}$ ., three hours' incubation at  $37^\circ \text{C}$ . following one hour's sterilisation at  $60^\circ \text{C}$ . Each flask was inoculated with one loopful of a thin emulsion of *B. mesentericus* B, and incubated. The rate of growth was estimated by making sub-cultures at 0, 4, 6 and 8 hours' incubation. The sub-cultures were made by inoculating 0.5 c.c. of the experimental fluid into a melted agar tube at  $45^\circ \text{C}$ ., and pouring as a plate culture. A series of figures obtained in this way is given below:

Table V.

Flour	Primary count	4 hours	6 hours	8 hours
Wholemeal	450	4,020	25,300	—
90 % extraction	400	2,470	12,300	35,000
80 %     ,,	336	2,056	9,040	32,100
80 %     ,,	332	1,900	9,630	28,200
70 %     ,,	408	672	9,430	24,400

These figures suggest that wholemeal flour forms the best medium for growth of *B. mesentericus*, that 90 % and 80 % extraction flours are less favourable for development, and 70 % extraction flours are the least favourable of the flours examined. The differences between the 90, 80 and 70 % extraction flours are not great, but are always of the same order.



## SUMMARY.

The skins of grains, all flours and all bread contain bacteria belonging to the group *B. mesentericus*. The cultural characters of six types of *B. mesentericus* isolated from grains and flour are given. Five of these were also obtained from ropy (or sour) bread. None of these five types can be identified as corresponding to the organisms isolated by earlier workers on rope. Rope, or sourness, does not result from the presence of these bacteria unless conditions are such as to allow of great development. The factors determining development of rope in bread are (1) degree of infection, (2) moisture, (3) temperature, (4) reaction, and (5) composition of the flour.

A method of estimating the degree of infection of flours is given.

## REFERENCES.

- BEATTIE and LEWIS (1917). *Lancet*, II. 217.  
BIEL (1896). *Centralbl. f. Bact.* 2te Abt. II. 137.  
CLARKE and LUBS (1917). *Journ. of Bact.* II. 1, 109, 191.  
COLE and ONSLOW (1916). *Lancet*, II. 9.  
EFFRONT (1917). *Compt. Rend.* 415.  
FLÜGGE (1896). *Die Micro-Organismen*, 3rd ed. II. 198.  
JESSON-HANSEN (1911). *Compt. rend. du Lab. de Carlsberg*, x. 17.  
HENDERSON, L. J. (1918). *Science*, N.S. XLVIII. 247.  
HÜPPE (1884). *Mittheil. a. d. Kaiserl. Gesundheitsamt*, Saml. II. 367.  
KRATSCHMER and NIEMILOWICZ (1889). *Vierteljahrschr. f. Nahrungs- u. Genussmittel*, 305.  
KRIES, quoted by Vogel (*v. infra*).  
LAURENT (1885). *Bull. de l'Acad. Roy. des Sci. de Belgique*, S. 3, x. 765.  
JORDAN LLOYD and MCCREA (1917). *Report to the Food (War) Committee of the Royal Society* No. 48.  
SEMLER (5. vi. 1917). *British Baker*.  
SØRENSEN (1909). *Compt. rend. du Lab. de Carlsberg*, VIII. 1.  
UFFELMANN (1890). *Centralbl. f. Bacteriol. u. Parasitenk.* VIII. 481.  
VOGEL (1897). *Zeitschr. f. Hyg.* XXVI. 398.  
WATKIN (30. iv. 1906). *Journ. of Soc. of Chem. Indust.*

## THE PREVALENCE OF THYROID ENLARGEMENT IN AND ABOUT HEREFORD.

BY HERBERT E. DURHAM, Sc.D., M.B., B.C. (CANTAB.), F.R.C.S. (ENG.),  
*Hereford.*

UNDER the title of "The inference of local degeneracy from a comparison of the vital statistics of the people," the late Dr C. S. Morrison, Medical Superintendent of the County and City Asylum, Hereford, published a somewhat alarming account of the local conditions (*Journal of Mental Science*, vol. LIII, 1907, p. 795). After showing that the mean quinquennial rate of admissions to the Asylum had risen from 4·8 per 10,000 inhabitants in 1871–1875 to 7·0 in 1891–1895 and after a slight drop in the succeeding period to 7·2 in 1901–1905 (not including boarded-out patients in the latter case), he proceeds to remark upon the prevalence of thyroid insufficiency. His words may be quoted as follows: "From records available, I find that the number has risen from 2·5 % of the admissions in 1886–1895 to 10·5 % during the decennium 1896–1905, and last year we admitted 3·7 % males and 33·5 % females with goitre. Allowing for any personal equation and any fuller observation made in the last decennium, the increase is sufficiently striking to be recorded, and has, I think, an important bearing on the issues of degeneracy shortly commented on in this paper. When we remember that thyroid insufficiency is associated with cretinism and myxoedematous insanity at one end of its evil chain, and an enormous number of minor mental and nervous retrogressions at the other, the evidence that thyroid disease is more prevalent in a community than it used to be, becomes one of serious import. It comes as a stranger within the gates we should like to eject!"

It was in fact a matter of common knowledge that goitrous necks were common in the rural districts of parts of Herefordshire, but latterly there is evidence that the diseased condition has invaded the city itself. The object of this communication is to call attention to the seriousness of this invasion. It is hardly possible to ascertain by direct evidence when it began, but some suggestion will be made in the following paragraphs.

### STREET OBSERVATIONS.

The introduction of the fashion of low or open necks as a part or rather a deficiency of modern female attire made it possible to observe the condition of the necks of individuals as they passed one in the street. From this somewhat casual mode of observation, one was forced to the conclusion that thyroid enlargement was quite a common condition in Hereford, whilst similar appearances were rare or unseen in strolls through the streets of towns in other districts. Whilst in other places hardly a single instance of thyroid



derangement could be observed, in a similar time in Hereford a dozen or more cases could be counted. It soon became apparent that the condition was not only observable on market days, when the town is filled with folk from the countryside, but that the town dwellers themselves were affected in considerable proportion. In 1916 the picture was rather blurred by the immigration of several thousand women, who came to work in the local shell-filling factory.

#### RECRUIT EXAMINATIONS. DISTRIBUTION OF THE DISEASE.

It is difficult, nay hardly possible, to give any thorough account of the regions which are most affected in the locality. A medical practitioner of the neighbouring town of Leominster, when questioned on the subject replied that "every other girl, you saw there, had a large thyroid"; and this though no doubt somewhat of *façon de parler* shows that the condition is widely spread there. The medical examination for recruiting, with which I was for a time engaged, afforded an opportunity for obtaining more information on the subject; though of course from piecemeal material it is impossible to base any proportion to population. The "rake-over" of re-examinees in the early part of 1918 mostly produced very poor types of manhood; on some days I counted as many as 1 in 8, 1 in 10 and 1 in 11 individuals with marked thyroid enlargement. Questions were put to ascertain that the condition had not been acquired elsewhere, and only in one case did it appear that the disease was of extra-comital origin—a lad from Derbyshire in whom it was a "family condition." It is generally accepted that thyroid derangement is far more common amongst women than men, so that these large proportions amongst the male sex are of considerable significance. One gained the impression that it was especially from the northern and western parts of the county that the cases were domiciled; it was only after a considerable number of cases had passed through that one single one was recorded from the Ross district, and he asserted that he had always lived there.

#### *City cases.*

Eventually two lads were presented who gave clear accounts that the enlargements they had had been acquired and developed during residence in the city, which is confirmatory of the conclusion that many of the numbers of quite young affected girls that one sees in the city have acquired their derangement whilst domiciled there. One of these two lads of 18 years definitely stated that he had lived all his life in the city and that the swelling in the neck had appeared two years before (*i.e.* in 1916). The other also gave a history of continued residence in the city but could not give any date for the onset of the enlargement, a question which is of interest in connexion with changes in the water-supply. It is possible that a certain amount of statistical knowledge might be excerpted from the medical history sheets of the recruits, but unless special attention happened to have been given to the matter the records could hardly yield a true maximum.

## CAUSATION.

Tradition has associated a causal connexion between goitre and water supply. Later and more stringent observations have tended to establish such a relationship. Earlier observers thought that "hardness" of the potable water-supply had its influence or at any rate that some mineral constituent was the cause; later the theory was started that some bacterial influence was at work, and after being under a cloud for many years, the bacterial theory has been revived notably through the work of R. McCarrison (*Etiology of Endemic Goitre*, Milroy Lectures, Bale, Daniellson, Ltd., London, 1913, and *The Thyroid Gland*, Baillière, Tindall and Cox, London, 1917). It has been adequately proved that boiling the water, if thoroughly done, renders it innocuous; it has also been shown that filtration through an unglazed porcelain filter likewise removes the noxa, which indeed may be demonstrable in the residue removed by the filter; less effective filtering agents such as the Berkefeld diatomaceous filters are not able to hold back the active agent, which also is unable to pass a dialysing membrane. Injurious water has been rendered harmless by antiseptics such as Hydrogen peroxide. These facts point to the conclusion that there is either a contagium vivum or some chemical body which is sensitive to, or destroyed by, these detoxicating means. Three theses may be staged that goitre is caused: (1) by some mineral agency in the water, (2) by some bacterial agency through pollution of the source, (3) by the combined action of these two agencies.

From the fact that removal to another district in which the disease is not endemic leads to recovery, it may be concluded that the supposed living virus cannot have great power of survival within the body; the same may be said of recovery during continuance of domicile in the dangerous area, care being taken to drink boiled water only (cases of both sorts have come to my knowledge locally).

*Mineral agencies in the water.*

It is quite clear that mere "hardness" of water, that is an undue content of calcium and magnesium compounds, cannot in itself be the direct cause. It may however be stated that the water of the countryside wells is highly charged with these two elements. Thus a well water I examined in 1908 and again in 1912 showed:

Alkalinity to methyl orange 5·6 to 4·75 c.c. N/1 acid per litre	
Calcium (as CaO) "temporary"	0·133 per litre
"          "          "permanent"	0·057
<hr/>	
total 0·190	
(another observation 0·198)	
Magnesia (as MgO) "temporary"	0·008
"          "          "permanent"	0·0048
<hr/>	
total 0·0128	



Potassium and sodium } (combined chlorides) }	0.080
Sulphates (as H <sub>2</sub> SO <sub>4</sub> )	0.067
Chlorides (as Cl)	0.031

In 1912 the town service water, during use of the old filter beds, gave:

Alkalinity	...	...	...	1.4 c.c. N/1 per litre
Total CaO	...	...	...	0.0554
Total MgO	...	...	...	0.0005
H <sub>2</sub> SO <sub>4</sub>	...	...	...	0.0105

In 1908, soon after Dr Morrison drew attention to the matter, I made some endeavours to determine whether some other constituent might have escaped attention such as strontium, but limitation in facilities precluded any satisfactory concentration of fractions from so large a quantity of lime. In regard to other metals, it may be noted that the Old Red Sandstone soils of the neighbourhood contain easily demonstrable amounts of manganese and titanium.

Let us now turn from the kations to the anions. The importance of the thyroid in relation to the iodine metabolism of the body has long been recognised, and it is not impossible that other halogen elements may also be dealt with therein, perhaps at the expense of the iodine. Chlorine may be put on one side and perhaps also bromine, for prolonged administrations of bromides have not evoked any remark concerning thyroid derangement. Fluorine is left for consideration, and gives rise to some points of interest. It appears to be many years since any thought of connexion between fluorides and goitre has been entertained; on searching the literature I find that Rabuteau (Paris, Baillière, 1867, 8vo., p. 144) was quite assertive on the subject. Nicklès (*Comptes Rendus Acad. Sci.* 1857) stated that all waters containing dissolved bicarbonate of lime also contained fluorides of calcium; Répin (quoted by McCarrison) stated that all goitrous waters were heavily charged with carbonic acid. Hitherto the chief attention in regard to fluorides has been centred on the bones and teeth. Rabuteau gives the following distribution of goitre in or about 1866, which perhaps is worth reproducing as the work is probably not to be found in every library (the Roy. Coll. Surgeons, London, England, possesses it).

Estimated goitrous persons per 100,000:

Côte d'Or...	...	...	55	Bas Rhin...	...	...	164
Marne	...	...	70	Haut Rhin	...	...	288
S.-et-Oise	...	...	46	Vosges	...	...	410
S.-et-Marne	...	...	37	Ariège	...	...	734
S.-Inférieure	...	...	33	Hautes Alpes	...	...	951
Seine	...	...	7				

Beyond insisting on the high content of fluoride in the waters of Contrèxe-ville, he does not carry the matter to the extent of giving the abundance of the compounds of fluorine in the other regions. Much importance is credited to an experiment by Mauméné on a bitch which was given doses of fluoride

of potash for five months and which three years later still had an enlarged thyroid; the story is not quite clear, for the dog was apparently lost for a time, moreover no detailed examination of the organ appears to have been made.

Brandl and Tappeiner (*Zeitschr. f. Biologie*, N.S. vol. x, p. 518, 1891) made a number of detailed observations on a dog which received large doses of sodium fluoride over a period of eight months, but unfortunately no attention was paid to the thyroid or associated glands in their elaborate analyses. Abegg (*Handbuch. d. anorgan. Chemie*, iv, p. 25, 1913) quotes observations on cattle which had fed upon the sludge from spirit factories, in which fluorides were used as yeast purifiers, but attention appears only to have been paid to the derangements in the bones.

On the other hand, McCarrison states that hydrofluoric acid has been administered with satisfactory results as a remedial agent.

The amount of attention which has been given to fluorides is perhaps exemplified by their entire omission in the toxicologies of Dixon Mann, Wynter Blyth, and Taylor; Kobert gives a few paragraphs on the subject. The question whether fluorides or silicofluorides have any share in disturbing the normal course of the thyroid would appear to be unanswerable at present though it might well form a field for enquiry.

#### THE WATER-SUPPLY OF HEREFORD.

It is laid down by McCarrison that a polluted water-supply and possibly contact with polluted soil, form the means whereby goitre is acquired and spread. Since some indubitably, and many probably, city-acquired cases of thyroid enlargement have occurred, the question of the water-supply becomes one of importance, and may be dealt with both from the mineral and bacterial standpoint. So far as can be ascertained the disease used not to arise within the city, and it is only in the last few years that it has done so. The water has always been pumped up from the river Wye into storage tanks whence it passes through filter beds of gravel. At Hay, especially, and no doubt here and there on its course, the river is liable to pollution from the riparian inhabitants. Here it may be noted that Hereford is remarkably free from typhoid fever and that the few cases which have been treated there have always been importations from without; so that one line of evidence of pollution is absent.

In this connexion, I understand, some years ago action was contemplated with the view of putting restraint on the Hay authorities, but nothing so far has been done. As a matter of history rather than as an argument in the discussion, it may be mentioned that Hereford has enjoyed the reputation of having escaped, when other places were ravaged by cholera; on the one hand the water-supply in those days must have been rather different in character to that now obtaining, and on the other, the escape might be explicable on the same idea that was given to account for the freedom of Versailles from the disease when Sanarelli found water vibrios in the Versailles



water, which closely resembled the true cholera vibrio, and suggested specific immunisation. Another, but temporary, chance of pollution would have undoubtedly occurred from the workmen during the erection of the new filter beds.

*Filtration.* The old filter beds which were in action previously to 1911 were quite incapable of dealing with the amount of water which modern consumption entailed. The filtration and settling were quite perfunctory, thus entomostraca and ctenodril worms were frequently to be found in the service water; and I observed that a Doulton filter on a service tap required cleansing every few days from the sludge which collected in order to get a normal flow from it. The filtration material consisted of the local gravels, but the output was far too great to allow any prolonged contact with the material.

The new filter beds which were installed in 1911 allow of more complete filtration and settling; at the same time they afford more opportunity for contact with and dissolution from the filtration material, which as heretofore consists of the local gravels. So that we have the two propositions, (1) better anti-bacterial filtration and (2) onset of thyroid derangement becoming concomitant. If it is assumed that some mineral constituent, which might be absorbed from the local gravels, is the cause or an adjuvant factor of the thyroid trouble, it might be inferred that the present treatment of the river water results in making it similar to the rural well waters by affording closer and longer contact with the local gravels. Two other differences may also be noted in respect to the changes: (1) that, as the river water was found to be slightly plumbisolvant, an artificial addition is made to increase its lime hardness, and (2) an electrically driven pumping mechanism has been installed. It is hardly likely that either of these alterations would have the undesirable effect.

In respect to pollution of the water as the cause of the goitre, there is the contradictory condition of less probability of pollution associated with apparently higher incidence of the disease. The evidence that has been put forward of the successful results of the use of autogenous vaccines is not at all conclusive for it can hardly be maintained that such widely different organisms as Staphylococci, spore bearing and "colon" bacilli could have one and the same *specific* effect; supposing that some other fallacy in observation may not have been toward, it would have to be inferred that the vaccines were contaminated by some unrecognised specific agent, which McCarrison believes to be an anaerobic bacillus.

Perhaps it might be well to contemplate the possibility of interaction between pollution and mineral constituents. By means of bacterial action insoluble substances in the soil may be brought to a soluble form. Thus some experiments in which fresh soil, to which carbohydrate (sugar or starch) was added and then allowed to ferment by the aid of its own bacteria, showed notably more (three-fold to five-fold) soluble potash, silica and phosphoric acid than did the fresh untreated soil. (Carbohydrate manuring and the rubbish

heap, H.E.D., *Gardener's Chronicle*, 1919, p. 91.) Thus it is probable that bacterial pollution may affect the dissolved mineral constituents of the water proceeding from a given soil.

#### PERIOD OF EXPOSURE TO THE CAUSATIVE NOXA.

In experimental endeavours to produce thyroid enlargement positive results have been noted in comparatively short periods; thus McCarrison observed enlargement in goats after 64 days' exposure to water filtered through contaminated earth. In the case of naturally acquired trouble probably the period between exposure and apparent onset would be much longer. So far as concerns Hereford, since the date of the first appearance of the goitre in the city is not knowable, it is hardly possible to make any assumption as to the probable period. Taking the case of the recruit (cited above) and supposing that the alterations in the water-supply were the origin of his trouble and that the ensuing changes in its constitution were continuously exhibited, it would appear that the exposure from 1911 to 1916 had been necessary to evoke the visible thyroid enlargement; naturally, however, changes would have been going on long before any visual effect had become manifest. McCarrison insists on changes in the finer anatomy of the organ without gross hypertrophy. Again it must not be lost to sight that during and since the war period there may have been some increase of susceptibility through limitations and alterations in the dietary. And such influence may have been both direct in affecting the actual nature and variety of food, and indirect in regard to the economies in fuel in its preparation, here, particularly, for instance in the making of tea the water may have not undergone so much boiling as in former times.

#### SUSCEPTIBILITY.

In any epidemic or endemic condition perhaps the most interesting part is the consideration of the reason why some individuals, though apparently equally exposed, escape disease altogether or only suffer in minor degree. In the case of bacterial infections, much can be explained by unsuspected specific immunity, and in some other conditions we now know that extraordinarily minute quantities of certain nutritional factors are able to produce gross effects; thus in beri-beri tiny quantities of certain constituents of the diet are able to make the nerves resist the unknown neurotropic poison, which the writer believes will eventually prove to be of bacterial origin.

In regard to the question of thyroid derangement, if the theory of an absorption of products from the intestine holds sway, the nature of the bacterial flora of the intestinal contents of a given individual may happen to be of prime importance whether in the direction of refractoriness or of susceptibility; also the good functioning of such organs as are ordinarily concerned in the destruction or elimination of noxious absorbed products, and thereby shelter or expose the thyroid to undue extent. Whilst this latter



thesis will also apply to a mineral causation of the goitre, quite as a fantastic chain one might put down: (1) the thyroid has been thought to be concerned with the calcium metabolism; (2) fluorides act as secondary protoplasmic poisons as decalcifiers and thus upset the calcium balance. But of course the evidence is too flimsy to use this more than as an illustration hypothetically.

Whilst environment is directly concerned, heredity may play an indirect part in the assumption of an absorption from the colon; a somatic inheritance of certain coils or disposition of gut or mesentery might make some individuals more susceptible to delayed movement and consequent greater absorption.

#### BIOLOGICAL ASPECT.

Perhaps one of the more interesting aspects of the matter is the biological one. Goitrous conditions whilst not necessarily going to the extreme of cretin production, are likely to have an effect on the mental development of the succeeding generation, and one would have thought that nature would prescribe some attempt at concealment where such a condition existed. However it would seem that fashion in dress is more potent, and leads the female population to expose their deficiency to the view.

#### CONCLUSION.

There are indications that the progressive increase of goitrous conditions, which Dr Morrison recorded in the country districts, has reached the city of Hereford. There is therefore a strong case for advocating an exhaustive investigation into the causes of the malady by enquiring into the local conditions so that a more generalised endemicity may be avoided. Already the county is in no enviable state in regard to its lunacy statistics, and unless some active remedial steps are taken without undue delay the outlook engenders pessimism.

INDEX OF AUTHORS

	PAGE
BLOCH, C. E. Clinical investigation of Xerophthalmia and Dystrophy in Infants and Young Children ( <i>Xerophthalmia et Dystrophia Alipogenetica</i> ). (With Plates II—IV and 5 Charts) . . . . .	283
BROWNING, C. H. and KENNAWAY, E. L. Suggestions for a New Criterion of a Positive Wassermann Reaction based on an Analysis of 2334 Quantitative Tests. (With 3 Text-figures) . . . . .	87
BRUCE, SIR DAVID. Tetanus. Analysis of 1458 Cases, which occurred in Home Military Hospitals during the years 1914—1918. (With 12 Diagrams and 1 Curve) . . . . .	1
BUXTON, P. A. Carriage of Coliform Bacilli by the Oriental Hornet ( <i>Vespa orientalis</i> Fabr.) . . . . .	68
CLARK, A. B., <i>see</i> LLOYD	
CUMMING, R. H. and MATTICK, A. T. R. An Enquiry concerning the State of Cleanliness of Empty Milk Churns . . . . .	84
CUNNINGHAM, ANDREW and THORPE, B. A. The Milk Supply of the City of Edinburgh	107
DUDGEON, LEONARD S. Blackwater Fever . . . . .	208
DURHAM, HERBERT E. The Prevalence of Thyroid Enlargement in and about Hereford . . . . .	394
FREDERICK, ROBERT C. The Conversion of Air into a Lethal Mixture of Gases by Storage of Tobacco and other Vegetable Substances . . . . .	205
GARDNER, A. D. A Valuation of the "Agglutinability-factor" in Dreyer's System .	333
GILL, C. A. Malaria in England, with Special Reference to the Rôle of Temperature and Humidity . . . . .	320
GRABHAM, GEORGE WALTER. Some factors in Thermal Sanitation in the Tropics. (With Plate I, 3 Charts and 3 Text-figures) . . . . .	245
GRAHAM-SMITH, G. S. The Behaviour of Bacteria in Fluid Cultures as indicated by Daily Estimates of the Numbers of Living Organisms. (With 29 Charts and 1 Text-figure) . . . . .	133
GREEN, IONE H. Report of Experiments on the Cold Storage of Herrings carried out at North Shields . . . . .	75
GRIFFITH, A STANLEY. A Study of the Serological Reactions of Meningococci and an account of the Method of Preparation of Anti-meningococcus Serum. (With 2 Charts) . . . . .	33
HEHEWERTH, F. H. and KOP, W. A. The Wassermann Test in Patients affected with Malaria in the Tropics . . . . .	277
HOPPE, E., <i>see</i> MALTANER	
KENNAWAY, E. L., <i>see</i> BROWNING	
KOP, W. A., <i>see</i> HEHEWERTH	
LLOYD, D. JORDAN, CLARK, A. B. and MCCREA, E. D. On Rope (and Sourness) in Bread. Together with a Method of estimating Heat-resistant Spores in Flour .	380
MCCREA, E. D., <i>see</i> LLOYD	



	PAGE
McDOWALL, R. J. S. The Water Supply of the Egyptian Expeditionary Force, with Special Reference to the Efficiency of Mechanical Rapid Filtration with Chlorination . . . . .	305
MALTANER, FRANK and HOPPE, ELLA. The Chemotactic Effect of Osmosis upon Leucocytes . . . . .	309
MATTICK, A. T. R., <i>see</i> CUMMING	
PRATT-JOHNSON, J. The Distribution of Malaria in South Africa and a Mosquito Survey of Military Hospital Areas . . . . .	344
SHEARER, C. On Defense Rupture and the Antagonistic Action of Salts . . . .	72
THORPE, B. A., <i>see</i> CUNNINGHAM	
TOPLEY, W. W. C. The Spread of Bacterial Infection. Some Characteristics of Long-continued Epidemics. (With 5 Charts) . . . . .	350
WILSON, W. JAMES. The Wilson-Weil-Felix Reaction in Typhus Fever . . . .	115
<i>Notice:</i> The Second International Congress of Comparative Pathology. (To have taken place in Rome, April, 1921, but now postponed indefinitely) . . . .	131

## INDEX OF SUBJECTS

	PAGE
"Agglutinability factor" in Dreyer's system . . . . .	333
Air vitiated by storage of tobacco, etc. in closed spaces . . . . .	205
Bacteria, behaviour in fluid cultures . . . . .	133
Bacterial infection, experiments on mode of spread . . . . .	350
Blackwater fever . . . . .	208
Bread, rope and sourness in . . . . .	380
Chemotactic effect of Osmosis on leucoeytes . . . . .	309
Defence rupture and antagonistic action of salts . . . . .	72
Dystrophy in infants and children . . . . .	283
Food preservation, <i>see</i> Herrings . . . . .	
Food, <i>see</i> Milk, Bread . . . . .	
Heat absorption by different kinds of cloth . . . . .	245 <i>et seq.</i>
,,    ,,    paints and building materials . . . . .	254
Herrings, experiments on cold storage of . . . . .	75
Immunity, <i>see</i> Tetanus, Meningococci, Defence rupture, Wassermann, Typhus, Chemotactic, Agglutinability, Bacterial	
Infection, <i>see</i> Bacterial . . . . .	
International Congress of Comparative Pathology ( <i>notice</i> ) . . . . .	131
Malaria in England, influence of temperature and humidity . . . . .	320
Malaria in South Africa and a mosquito survey . . . . .	344
Malaria, Wassermann test in malaria patients . . . . .	277
Malnutrition, <i>see</i> Xerophthalmia, Dystrophy . . . . .	
Meningococci, serological reactions and antisera for . . . . .	3
Milk churns, enquiry regarding cleanliness of . . . . .	84
Milk supply of Edinburgh . . . . .	107
Mosquitoes, <i>see</i> Malaria . . . . .	
Tetanus in Home Military Hospitals 1914-1918 . . . . .	1
Thyroid enlargement, prevalence about Hereford . . . . .	394
Tropical sanitation, <i>see</i> Heat . . . . .	
Typhus fever, Wilson-Weil-Felix reaction in . . . . .	115
<i>Vespa orientalis</i> Fabr. as carrier of coliform bacilli . . . . .	68
Wassermann reactions . . . . .	87
Wassermann reactions, <i>see</i> Malaria . . . . .	
Water supply of Egyptian army (filtration, chlorination) . . . . .	305
Xerophthalmia in infants and children . . . . .	283









RA

421

J88

v.18-19

cop.2

The Journal of hygiene  
vol.18-19

Biological  
& Medical  
Serials

PLEASE DO NOT REMOVE  
CARDS OR SLIPS FROM THIS POCKET

---

UNIVERSITY OF TORONTO LIBRARY

---

